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**Studies on the virulence factor involved in the pathogenicity
in tick-borne encephalitis virus infection**

(ダニ媒介性脳炎ウイルス感染における病原性発現に関与するウイルス側因子に関する研究)

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ABBREVIATIONS

BHK	: baby hamster kidney
C	: core
DIG	: digoxigenin
dpi	: days post-infection
dsRNA	: double-stranded RNA
E	: envelope
FCS	: fetal calf serum
hpi	: hours post-infection
IC	: infectious clone
IFN	: interferon
IRF	: interferon regulatory factor
ISGs	: interferon-stimulated genes
ISRE	: interferon-stimulated regulatory element
JAK-STAT	: janus kinase-signal transducer and activator of transcription
LD ₅₀	: 50% lethal dose
MEM	: minimum essential media
MOI	: multiplicity of infection
NS	: non-structural protein
nt	: nucleotide
ORF	: open reading frame
PBS	: phosphate buffered saline
pfu	: plaque-forming unit
prM	: precursor membrane
PRRs	: pattern recognition receptors
pt	: parent
RIG-I	: retinoic acid inducible gene-I
rpm	: revolution per minute
sfRNA	: subgenomic flavivirus RNA
SL	: stem loop
TBE	: tick-borne encephalitis
TBEV	: tick-borne encephalitis virus
TLR	: toll-like receptor
UTR	: untranslated region
XRN1	: exoribonuclease 1

INTRODUCTION

Tick-borne encephalitis (TBE) virus, which is a member of the genus *Flavivirus* in the family *Flaviviridae*, causes fatal encephalitis in humans. It is a major arbovirus that causes thousands of cases of severe neurological illness annually [1]. TBE is a significant public health problem in endemic areas of the European and Asian countries [2, 3]. Mortality rates vary from about 0.5 to 30%, and neurological sequelae can occur in 30 to 60 % of survivors [4-6]

TBE virus (TBEV) is a positive-stranded RNA virus with a genome of ~11 kb that encodes a long polyprotein in a single open reading frame (ORF), flanked by the 5'- and 3'-untranslated regions (UTRs). The corresponding polyprotein is processed into structural proteins, i.e., capsid (C), pre-membrane (prM), envelope (E) protein, as well as non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [7, 8]. The C protein is associated with the genome RNA packaging and neuropathogenesis of TBEV [9, 10]. The M protein is initially translated as a precursor protein known as prM [11], forms a heterodimer with the E protein, and functions to protect the E protein. It is known that the E protein is responsible for binding to cellular receptors, tissue tropism and virulence, as well as for the induction of virus-neutralizing antibodies [12-14]. The non-structural proteins play roles in the genome replication and the processing of viral proteins. NS3 functions as a protease [15, 16], and helicase [17], and NS5 functions as a methyltransferase [18], RNA-dependent RNA polymerase, and interferon antagonist [19]. The 5'- and 3'-UTR are believed to be associated with the viral genome replication [20, 21].

The 3'-UTR of TBEV is divided into two domains; the 5'-terminal variable region

and 3'-terminal core element. The core element is highly conserved among TBEV strains. It's reported that this element contains a sequence that is essential for the viral genome replication [21].

The sequence and length of the variable region vary among TBEV strains. The variable region is considered that it's not involved in the ability of viral replication and virulence in mice [22]. A deletion or insertion of polyA sequence exists in the variable region of some strains [23]. Strains isolated from ticks and wild rodents do not contain a deletion or insertion of polyA sequence in the variable region, and this region is considered to be essential for the natural transmission cycle of TBEV [24]. In contrast, deletions or insertion of polyA sequence in the variable region were found in strains passaged in mammalian cell culture [22]. Deletions were also observed in the TBEV strains isolated from human patients [22, 25, 26]. These reports suggest that the deletion or the insertion of polyA sequence is caused by viral adaptation to mammalian cells and is related to the viral virulence causing severe case in human. However, the role of this region remains unclear.

Recently, it's reported that subgenomic flavivirus RNA (sfRNA) is produced as the product of degradation of viral genomic RNA in flavivirus infection [27, 28]. The production of sfRNA is caused by one of the host exoribonuclease, XRN1. It's believed that sfRNA is involved in the virus replication in cultural cells and virulence in mice by interfering with host protective responses, such as RNA interference machinery and type I interferon (IFN) reaction [28].

IFN system is an important mechanism of the host's first line of defense system against virus infections [29-33]. Type I IFN is induced through the recognition of viral signature molecules including double-stranded RNA (dsRNA), by pattern recognition

receptors (PRRs), such as the cytoplasmic retinoic acid-induced gene-I (RIG-I) family or the membrane-bound Toll-like receptors (TLRs) [34, 35]. It's reported that type I IFN response has a critical role in the host's defense against TBEV [36]. Some studies showed that the NS5 protein of TBEV has a role of IFN antagonism by inhibition of janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling [37].

Based on phylogenetic analysis, TBEV can be divided into three subtypes: the Far-Eastern, European and Siberian subtype[4]. Each subtype causes different symptoms and mortality [2, 4]. The Far-Eastern subtype is also known as Russian spring summer encephalitis virus, and is prevalent in Far-Eastern Russia. This subtype causes severe neural disorders such as encephalitis and meningoencephalitis with a higher mortality rate up to 30 % [24, 38]. The European subtype produces biphasic febrile illness and milder encephalitis, and the mortality rate is lower than 2% [39]. The Siberian subtype also causes less severe disease (case mortality rate, 7 to 8%) than the Far-Eastern subtype and is often associated with chronic disease [4].

The virus strain Sofjin-HO was isolated from a patient in Russia in 1937 and has been used as a prototype of the Far-Eastern subtype [40]. It is also known to be highly pathogenic in a mouse model. The strain Oshima 5-10 was isolated from a sentinel dog in 1995 in the area in which a human case of TBE was reported in Japan, and was classified as the Far-Eastern subtype of TBEV. Oshima 5-10 is less virulent than Sofjin-HO in a mouse model [24, 41, 42]. The nucleotide homology between Oshima 5-10 and Sofjin-HO is high (96%) with differences of only 44 amino acids and a deletion of 207 nucleotides in the 3'-UTR of Sofjin-HO (Table 1). However, no information exists concerning the detailed mechanisms of different virulence in the two closely related

strains although they exhibit a high homology. Identifying the genetic factors associated with the different virulence is expected to facilitate elucidation of the mechanism of pathogenicity of TBEV.

Infectious cDNA clones are useful in investigating the genetic determinants of flavivirus replication and pathogenicity. Infectious cDNA clones have been generated for multiple flaviviruses, including yellow fever virus, West Nile virus, Dengue virus, Japanese encephalitis virus, Omsk hemorrhagic fever virus and TBEV [24, 43-47]. In previous studies, our group constructed full-length infectious cDNA clones of the Far-Eastern subtype Oshima 5-10 and Sofjin-HO strains [48-50].

In the present study, I constructed recombinant viruses between the infectious cDNA clones of the Far-Eastern subtype Sofjin-HO and Oshima 5-10 strains. The virulence of the chimeric viruses was subsequently investigated in a mouse model. I showed that the 3'-UTR variable region is an important factor that determines the virulence of the Far-Eastern subtype of TBEV and tried to evaluate how the region is related to the pathogenicity of TBEV infection.

MATERIALS AND METHODS

Cells

Baby hamster kidney (BHK)-21 cells and mouse neuroblastoma NA cells were grown in Eagle's minimal essential medium (E-MEM), supplemented with 8 and 10% fetal calf serum (FCS), respectively. Human neuroblastoma SYM-1 cells were kindly provided by Dr. A. Kawai (Research Institute for Production Development, Kyoto, Japan), and maintained in E-MEM supplemented with 10% FCS.

Viruses

Viruses were prepared from infectious cDNA clones. Infectious cDNA plasmids of parental Sofjin-IC and Oshima-IC (Sofjin-IC-pt and Oshima-IC-pt), which encode the full-length cDNA of the TBEV Sofjin-HO (GenBank accession no. AB062064) and Oshima 5-10 (GenBank accession no. AB062063) strains, respectively, were prepared as described previously [48, 50].

Infectious cDNA plasmids of the recombinant viruses listed in Figs 2a and 3a were constructed by the replacement of the indicated regions between Sofjin-IC-pt and Oshima-IC-pt using the indicated restriction enzyme sites. To construct infectious cDNA plasmids of the recombinant viruses listed in Fig. 4a, the DNA fragments with the indicated nucleotides of Sofjin were amplified by fused PCR and were inserted into Oshima-IC using the *Age* I and *Aat* II restriction enzyme sites. Oshima-IC/sofjinNS2A^C was constructed by site-directed mutagenesis as described below. To construct infectious cDNA plasmids of recombinant Oshima-IC viruses with substitutions of

single amino acids, site-directed mutations were introduced into position 225 of NS2A, and positions 778, 827, 832 and 862 of NS5 using the Quick Change II XL site-directed mutagenesis kit (Agilent Technologies, CA, USA) as shown in Figs 3a and 4a.

To construct infectious cDNA plasmids of the recombinant virus Oshima-IC/sofjin3'-UTR_vari (Oshima_dLoop2/3/4) and Oshima-IC/sofjin3'-UTR_core, the fragment of the variable region (nucleotide 10377–10551) and the core element (nucleotide 10552–10894) of Sofjin-IC were amplified by fused PCR, and were inserted into Oshima-IC using *Asc* I and *Spe* I, as shown in Fig. 5a. The differences of nucleotides and amino acids between Sofjin, Oshima and each recombinant virus are shown in Table 1.

To construct infectious cDNA plasmids of the recombinant virus Oshima_dSL2/3, Oshima_dSL4, and Oshima_polyA, the fragments with the deletion of nucleotide 10443-10567 or 10568-10649, or with insertion of 35 adenines between 10493 and 10494 respectively, were amplified by fused PCR, and inserted into Oshima-IC using *Asc* I and *Spe* I (Fig. 7a).

The infectious cDNA plasmids were linearized with *Spe* I and were transcribed into RNA using the mMMESSAGE mMACHINE SP6 Kit (Life Technology, CA, USA) as described previously [51]. The mRNA samples were treated with DNase I and precipitated with LiCl. The precipitated RNA was dissolved in 30 µl diethyl pirocarbonate (DEPC) -treated water. BHK-21 cells were transfected with mRNA using a TransIT-mRNA Transfection Kit (Mirus Bio, WI, USA) as described previously [49]. Two days post-transfection (dpi), recombinant viruses in the supernatant of the

RNA-transfected cells were harvested and were stored at -80 °C. All experiments using recombinant viruses were performed according to the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms of Japan, a Japanese law ensuring compliance with the Cartagena Protocol on Biosafety. All experiments using live viruses were performed in a BSL-3 facility.

Virus titration

Plaque assays were carried out with BHK-21 cells using 12-well plates. The cells were inoculated with serial 10-fold dilutions of organ suspensions or culture medium from infected cells (100 µl), and they were incubated for 1 hour at 37 °C before 1.5% carboxy methyl cellulose in MEM (1 ml/well) was added. Incubation was continued for 3-4 days and the monolayers were stained with 0.1% crystal violet in 10% formalin neutral buffer solution. Plaques were counted and infectivity titers were expressed as plaque-forming unit (pfu) /ml.

Growth curve in cell culture

Subconfluent NA cells were grown in 24-well plates. Cells were inoculated with each virus at a multiplicity of infection (MOI) of 1. Cells were incubated at 37 °C in 5% CO₂. The supernatant was harvested at 8, 16, 24 and 48 hours post-infection (hpi) and stored in aliquots at -80 °C.

Animal model

Five-week-old female C57BL/6 mice (Jackson ImmunoResearch, PA, USA) were

inoculated subcutaneously with 1,000 pfu of virus. Morbidity was defined as the appearance of 10% weight loss. Surviving mice were monitored for 28 dpi to obtain survival curves and mortality rates. For the analysis of viral distribution in tissues, serum, brain and spleen were collected from the mice at 1, 3, 5, 7 and 9 dpi. Organs were individually weighed and homogenized, and prepared as 10% suspensions (w/v) in PBS with 10% FCS. The suspensions were clarified by centrifugation (4,000 rpm for 5 min, 4 °C) and the supernatants were titrated by plaque assay on BHK-21 cells. All procedures were performed according to the guidelines of the Animal Care and Use Committee of Hokkaido University.

Histopathological examination

Three mice subcutaneously infected with 10^3 pfu of TBEV were killed at 7 and 9 dpi, and formalin-fixed brains were routinely processed and embedded in paraffin, sectioned, and stained with haematoxylin and eosin as described previously [52].

Immunohistochemical detection of TBEV antigens was performed using rabbit polyclonal antibodies against E protein to detect TBEV antigens (1:500) [53].

Northern Blotting

Total RNA was extracted with ISOGEN (NIPPON GENE, TOKYO, Japan) following the manufacturer's recommendations. The RNA (4 µg) was subjected to denaturing gel electrophoresis in a 2% agarose and 2% formaldehyde gel followed by transfer onto Hybond-N membranes (GE Healthcare, Buckinghamshire, UK).

Pre-hybridization and hybridization were carried out in digoxigenin (DIG) Easy Hyb solution (Roche Diagnostics, Basel, Switzerland) at 68 °C by using 0.5 ng/ml of positive or

negative sense DIG-labeled (Roche Diagnostics) RNA for 3'-UTR (nucleotide 10277-11100 of Oshima-IC-pt). After hybridization, the membranes were blocked and washed with DIG Wash and Block Buffer Set (Roche Diagnostics). The bound probes were reacted with alkaline phosphatase conjugated anti-DIG Fab fragments (Roche Diagnostics), and the bands were visualized with CDP-Star (Roche Diagnostics).

Transfection and reporter assay

Transfection was performed by using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics) with according to the manufacturer's instructions. NA cells and SYM-1 cells were transfected with 1 µg of pISRE-Luc Cis-Reporter Plasmid (Agilent Technologies) or 4×IRF-3-Luc reporter plasmid (kindly provided by Dr. Rongtuan Lin, Jewish General Hospital-McGill University, Montreal, Canada), respectively. Four copies of the IRF-3-binding positive regulatory domain (PRD) I/III motif of the IFN-β promoter are contained upstream of the luciferase reporter gene in the 4×IRF-3-luc plasmid. At 24 hpi, the cells were inoculated with Oshima-IC-pt, Oshima/dSL2/3/4 or Sofjin-IC-pt at a MOI of 1, respectively. To activate the ISRE promoter, NA cells were treated with 500 U/ml mouse interferon (IFN) -α (PBL Assay Science, NJ, USA) at 24 hpi, and three hours after the treatment, the cells were collected. The SYM-1 cells were collected at 48 hpi. The Cells were lysed and the luciferase activities were determined by Luciferase Assay Systems (Promega, WI, USA) according to the manufacturer's instructions. All assays were carried out in triplicate, and the results expressed as means ± the SD.

Statistical analysis

P values of differences in virus titers were calculated using an unpaired Student's *t* tests.

RESULTS

Replacement of the coding region for the structural proteins had no effect on virulence

The structural proteins of flaviviruses, especially the E proteins, have been reported to be important for virulence [12-14]. To examine whether the structural proteins are determinants of virulence in mice, Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME were constructed by replacement of the coding region for most of the structural proteins (nucleotide 240-2291) with that of Oshima-IC-pt and Sofjin-IC-pt, respectively (Fig. 2a). Although relatively lower growth was observed in the chimeric viruses, infectious viruses were recovered (Fig. 2b). The pathogenicity of the recombinant viruses was examined in a mouse model. C57BL/6 mice were infected subcutaneously with 1,000 pfu of Sofjin-IC-pt, Oshima-IC-pt, Sofjin-IC/oshimaCME or Oshima-IC/sofjinCME virus, and survival was recorded for 28 days. The mice inoculated with each virus showed general signs of illness, such as reduced body weight, ruffled fur, and neurological signs of trembling and hind-limb paralysis; however, the survival time was longer and the mortality rate was lower in the mice infected with Oshima-IC-pt than in those infected with Sofjin-IC-pt (Fig. 2c, Table 2). The viruses in which the coding region for the structural protein were replaced (Sofjin-IC/oshimaCME or Oshima-IC/sofjinCME) showed virulence similar to that of the parental Sofjin-IC-pt or Oshima-IC-pt, regarding the survival curve, mean survival time and mortality in mice (Fig. 2c, Table 2). The results suggested that the difference in virulence between Sofjin and Oshima strains was not due to the structural proteins.

C-terminus of NS5 and the 3'-UTR are associated with the difference in virulence between Sofjin and Oshima strains

As the replacement of the coding region for structural proteins did not affect the virulence, the other regions were investigated next. Recombinant Oshima-IC viruses were generated by partial replacement of the regions except the coding sequence for structural proteins, as shown in Fig. 3a. The growth of each chimeric virus was higher than that of the parental Oshima strains (Fig. 3b). Mice were then infected with these recombinant viruses. Compared with the Oshima-IC-pt virus, mice infected with each recombinant virus showed a higher mortality and shorter survival time (Fig. 3c, Table 2). However, only mice infected with the Oshima-IC/sofjin NS5^C-3'-UTR virus, in which the coding regions for the C terminus of NS5 and 3'-UTR were replaced with those of Sofjin-IC, showed a similar virulence to that of mice infected with Sofjin-IC-pt, regarding the survival curve, days of onset, mean survival time (significantly shorter than that of Oshima-IC-pt) and mortality (Fig. 3c, Table 2). These results suggested that the C terminus of NS5 and/or the 3'-UTR was important for the difference in virulence between the Sofjin-HO and Oshima5-10 strains (Table 1).

As Oshima-IC/sofjin NS2A^C-4B^N also showed a high pathogenicity with a short survival time, recombinant Oshima-IC viruses with replacement of the genes for NS2A, the N-terminal or C-terminal region of NS3, or the N terminus of NS4B were constructed (Fig. 4a). No difference was noted in the amino acids of NS2B and NS4A between the Sofjin-HO and Oshima 5-10 strains. The growth of each chimeric virus was almost similar to that of the parental Oshima strain (Fig. 4b). The mortality of mice was 100% following infection of the chimeric virus with the N-terminus of NS3. However, compared with Sofjin-IC-pt, the days to onset and survival time were longer in mice

infected with the chimeric virus. The mice infected with the other viruses showed survival curves similar to the mice infected with Oshima-IC-pt, and no significant difference was found in the mean survival time between each virus and the parental Oshima-IC-pt. These results indicated that the difference in virulence between the Sofjin and Oshima strains could also be attributed to the N-terminus of NS3, which encodes a serine protease.

Partial deletion of the variable region of the 3'-UTR affects virulence

There are 4 amino acid differences in the C-terminus of NS5 and the nucleotide differences in the 3'-UTR. The 3'-UTR can be divided into two regions; the variable region which varies among TBEV strains, and the core element which is highly conserved in its sequence. In the variable region, there are 2 nucleotide differences between the Sofjin and Oshima strains, and a deletion of 207 nucleotides is present in the variable region of Sofjin, as shown in Fig. 5a. In the core element, 12 nucleotide differences are evident between the two strains. To identify the factor(s) that affects the virulence in the coding regions for the C-terminus of NS5 and the 3'-UTR, I constructed recombinant Oshima-IC viruses with a single amino-acid substitution in NS5 and replacement of the variable region or core element of the 3'-UTR, as described in Fig. 5a. The virus titer of the supernatant of Sofjin-IC-pt infected cells was significantly higher than that of each chimeric virus or Oshima-IC-pt infected cells. No significant difference in each chimeric virus and Oshima-IC-pt viral titers was found (Fig. 5b). Mice were then infected with each recombinant virus. Only the Oshima-IC/sofjin3'-UTR_vari virus, in which the variable region was replaced with that of Sofjin-IC-pt, killed 100% of mice and showed almost identical virulence to that of Sofjin-IC-pt virus, in terms of the

survival curve, days of onset and mortality. Conversely, the other recombinant viruses showed a similar virulence to that of the mice infected with Oshima-IC-pt (Fig. 5c, Table 2). These results suggested that the deletion in the variable region of the 3'-UTR is an important determinant of the difference in virulence between the Sofjin and Oshima strains.

Effect of the deletion of the stem loop structures in the variable region of the 3'-UTR on viral multiplication and pathogenicity

The RNA secondary structures of the variable region of the Sofjin and Oshima strain were predicted by RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (Fig. 6). The variable region of Oshima has six stem loop (SL) structures (SL1 to SL6), while the region including the SL2, SL3 and SL4 was deleted in Sofjin. To confirm whether the specific RNA sequence or secondary structure of the region decides the different virulence between Sofjin and Oshima, I next constructed Oshima-based mutant viruses with the deletion of the SL2, SL3 and SL4 (Oshima-dSL2/3/4), SL2 and SL3 (Oshima-dSL2/3), or SL4 (Oshima-dSL4) (Fig. 7a). A recombinant virus with the insertion of polyA sequence (Oshima_polyA) was also constructed to investigate the effect of the insertion as seen in the other strains with high passage history in cultural cells (Fig. 7a), such as Neudoerfl strain of the European subtype. The growth of the mutant viruses were significantly lower than Sofjin-IC-pt and it was same level with Oshima-IC-pt in NA cells (Fig. 7b). This result suggested that the partial deletion or the insertion of polyA sequence did not affect viral multiplication in cultural cells.

The mutant viruses and parental viruses were infected into mice. All mutant viruses with partial deletion or insertion of polyA sequence showed almost same

virulence with Sofjin in the manner of survival curve, days of onset and mortality (Fig. 7c, Table2). There was no difference in the increased virulence by the each partial deletion in the SL2 to SL4 structures of Oshima_dSL2/3 and Oshima_dSL4, indicating that the both of the partial deletion of the region of SL2/3 and SL4 increased the virulence in mice.

To examine the correlation between disease development and viral replication in organs, the viral loads in the blood, spleen and brain were compared in mice inoculated with the Sofjin-IC-pt, Oshima_dSL2/3/4, Oshima_dSL2/3, Oshima_dSL4, Oshima_polyA or Oshima-IC-pt viruses (Fig. 8). Transient viremia was observed in the mice infected with each virus, which almost disappeared by 5-7 dpi. Increases in viral replication were observed in the spleen after viremia (from 3 dpi). Compared with Oshima-IC-pt, the viral titer in blood was lower in the mice infected with the other viruses with the deletions or insertion. The viruses were detected in the brain by 7 dpi in the mice inoculated with Sofjin-IC-pt, the titers reached 1.1×10^8 pfu/ml and 3.4×10^9 pfu/ml at 7 and 9 dpi, respectively, and were significantly higher than that in the mice infected with each Oshima-based viruses or Oshima-IC-pt ($p < 0.05$). No significant difference was observed between titers in the brains infected with the each mutant viruses and Oshima-IC-pt.

Histopathological features in the brains of mice infected with Oshima- IC-pt, Sofjin-IC-pt or Oshima_dSL2/3/4 at 7 and 9 dpi were investigated (Fig. 9). At day 7, there were few pathological changes in mice infected with each virus (data not shown). However, at day 9, abundant viral antigens and pathological changes, such as inflammatory cell infiltrations, small haemorrhages and necrotic or degenerated neurons, were observed throughout the brains of mice infected with Sofjin-IC-pt and

Oshima_dSL2/3/4. Compared with Sofjin-IC-pt⁻ and Oshima_dSL2/3/4-infected mice brains, there were fewer virus-antigen positive cells and mild pathological changes in the brains of mice infected with Oshima-IC-pt.

Taken together, these data suggested that the deletion in the variable region of the 3'-UTR did not increase virus multiplication, but enhanced the virulence in the mouse brain.

Effect of the deletion in the variable region on the sfRNA production and IFN antagonism

It has been reported that 3'-UTR is related to production of subgenomic flavivirus RNA (sfRNA). sfRNA has been shown to regulate pathogenicity and innate immunity in some flaviviruses infection, such as West Nile virus, Japanese encephalitis virus and so on. I performed northern blotting analysis to confirm whether sfRNA is produced during the infection of the Far-Eastern subtype of TBEV. As shown in Fig. 9, two different sizes of sfRNAs (about 300 or 500 base) were produced in the infected BHK-21 cells (Fig. 10). However, among the viruses, there was no difference in the size of sfRNA and ratio of amount of sfRNA to genomic RNA (data not shown). This result suggested that the deletion or insertion of polyA sequence in the variable region did not affect the production of sfRNA, and that they were not directly associated with the different pathogenicity between Sofjin and Oshima strains.

I next investigated the effect of the deletion or insertion of polyA sequence in the variable region on the induction of interferon (IFN)- β and interferon-stimulated genes (ISGs) by reporter assay (Fig. 11). The promoter activity of IFN- β was significantly higher in cells infected with Sofjin-IC-pt than Oshima-IC-pt and the

Oshima-dSL2/3/4. The interferon-stimulated regulatory element (ISRE) promoter activity was lower in the cells infected with Sofjin than those infected with Oshima-based virus. No difference was observed by the deletion or the insertion of RNA sequence in the variable region.

These results suggested that the deletion in the variable region affected neither the sfRNA production nor the induction of the type I IFN and ISGs.

DISCUSSION

In the present study, the important determinants of virulence were identified between the Far-Eastern subtype Sofjin and Oshima strains of TBEV. I showed that multiple viral factors affected the virulence cumulatively and that the variable region of the 3'-UTR was a critical virulence determinant.

The E protein is thought to play a key role in determining the virulence of TBEV [54]. The E protein is expressed on the surface of mature virions and mediates virus entry into the host cell by binding to cell surface molecules [7]. The E protein has been suggested to be a crucial determinant of tissue tropism and neuropathogenesis during flavivirus infection. Amino acid changes in the E protein have been reported to affect the neurovirulence and neuroinvasiveness of tick-borne flaviviruses [14, 55-57]. However, the structural proteins, including the E protein, were not associated with the different virulence between the Sofjin-HO and Oshima 5-10 strains.

Replacement of the N-terminus of NS3 increased virulence in mice. The flavivirus NS3 encodes a serine protease domain at its N-terminal that is required for cleavage of the polyprotein during viral replication [58]. It combines with NS2B and forms the NS2B-NS3 protease complex as the activated serine protease [15, 59, 60]. Several amino acid substitutions in the protease domain of NS3 can influence the activity of the enzyme and the virulence of TBEV [42, 61, 62]. Seven amino acid differences in the N-terminus of NS3 exist between Sofjin-HO and Oshima 5-10 strains. A previous report suggested that the serine to phenylalanine substitution at position 45 affects TBEV pathogenicity [42]. An identical substitution was also observed between the Sofjin-HO and Oshima 5-10 strains. Therefore, this substitution might be associated with partially

affecting the difference in virulence between Sofjin-HO and Oshima 5-10 strains.

The 3'-UTR of TBEV consists of two distinct domains: the 5'-terminal variable region and 3'-terminal core element [63, 64]. The core element shows a high degree of sequence conservation among TBEV strains and contains sequences necessary for viral genome replication, such as cyclization sequence [21]. The sequence of the variable region varies among the TBEV strains and the role of this region is unclear. In a study of the European TBEV subtype strains, deletion of the entire 3'-UTR variable region did not affect viral multiplication in cultural cells or virulence in mice [22]. The discrepant results obtained in the present study might be due to the use of different strains. As the Neudoerfl strain used in the study of Mandl *et al* (1998) was highly virulent in the mouse model ($LD_{50}<10$), it is possible that deletion of the whole variable region did not result in an increase in the virulence. Additionally, the Neudoerfl strain contains an insertion of a polyA sequence in the variable region [22, 23] that is not present in most other TBEV strains.

The sequence of the 3'-UTR variable region varies among TBEV strains; however, the role of this region remains unknown. Strains freshly-isolated from ticks and wild rodents do not have a deletion in the variable region, and this region is considered to be essential for the natural transmission cycle of TBEV [24]. Conversely, deletions in the variable region of 3'-UTR were found in many Far-Eastern subtype isolates from human patients [25]. Mandl *et al* reported that the deletion in the 3'-UTR occurred during passage in mammalian cell culture or in mice [22]. Also, polyA sequence in this region cannot be found in strains isolated from natural host. The strain Oshima 5-10, which was isolated from a sentinel dog in nature with low passage history, doesn't have deletion in the variable region. On the other hand, the strain Sofjin-HO isolated from a

patient with high passage history has a deletion in the variable region. Together, these data suggest that the deletion caused by adaptation or selection in mammalian cells affected increased virulence in mammals.

Increased inflammatory responses and lesions in the brain were observed by the deletion in the variable region, although the deletion did not affect the viral multiplication in the brain. These data suggested that the variable region might have a role for the alteration of host responses in the brain. In this study, both of the partial deletion of SL2/3 and SL4 (non-overlapping region) increased the pathogenicity. It has been reported that several host proteins, such as La, p100, FBP1 and Mov34, bind to the 3'-UTRs of flaviviruses [65-70]. The La protein was shown to bind to 5'- and 3'-UTR of Japanese encephalitis virus and to affect the viral multiplication process [70-72]. It might be possible that the intact conformational structure which consists of SL2 to SL4 interacted with host factors involved in the pathogenicity. Reduction in viremia was observed by the deletion in the variable region. Some study showed that immune response in peripheral organ affected the viral pathogenicity in brain [73]. There is a possibility that the difference in the multiplication in viremia might affect the host peripheral immune response associated with the pathogenicity in brain.

A recent study of West Nile virus and Japanese encephalitis virus reported that the subgenomic flavivirus RNA (sfRNA) was produced from the 3'-UTR as a product of the genomic RNA degradation by host exoribonuclease XRN1 and that sfRNA mediated pathogenicity by interfering with host protective responses, such as the RNA interference machinery and type I IFN response [27, 74]. IFN response is one of the important innate immunity systems to clear viruses [29, 33, 75]. Some studies indicate that viral factors such as sfRNA and the NS5 protein of TBEV have IFN antagonist

activity [27, 28, 37]. In this study, the difference in the variable region between Sofjin and Oshima did not affect the production in the sfRNA production. Furthermore, the deletion in the variable regions did not affect the IFN- β or ISGs induction. These data suggested that unknown mechanism rather than sfRNA production or IFN antagonism might be involved in the increased pathogenicity by the conformational changes in the variable region.

In conclusion, I reported here that the variable region of the 3'-UTR is an important determinant of pathogenicity in mice. The deletion and insertion of polyA sequence in the region affected multiplication in periphery but not in brain, resulting in the severe pathological changes associated with the Far-Eastern subtype TBEV. These findings encourage further research to identify the pathogenicity of TBEV and develop prevention and therapeutic strategies for TBE.

Table 1. Amino acid differences between Sofjin and Oshima.

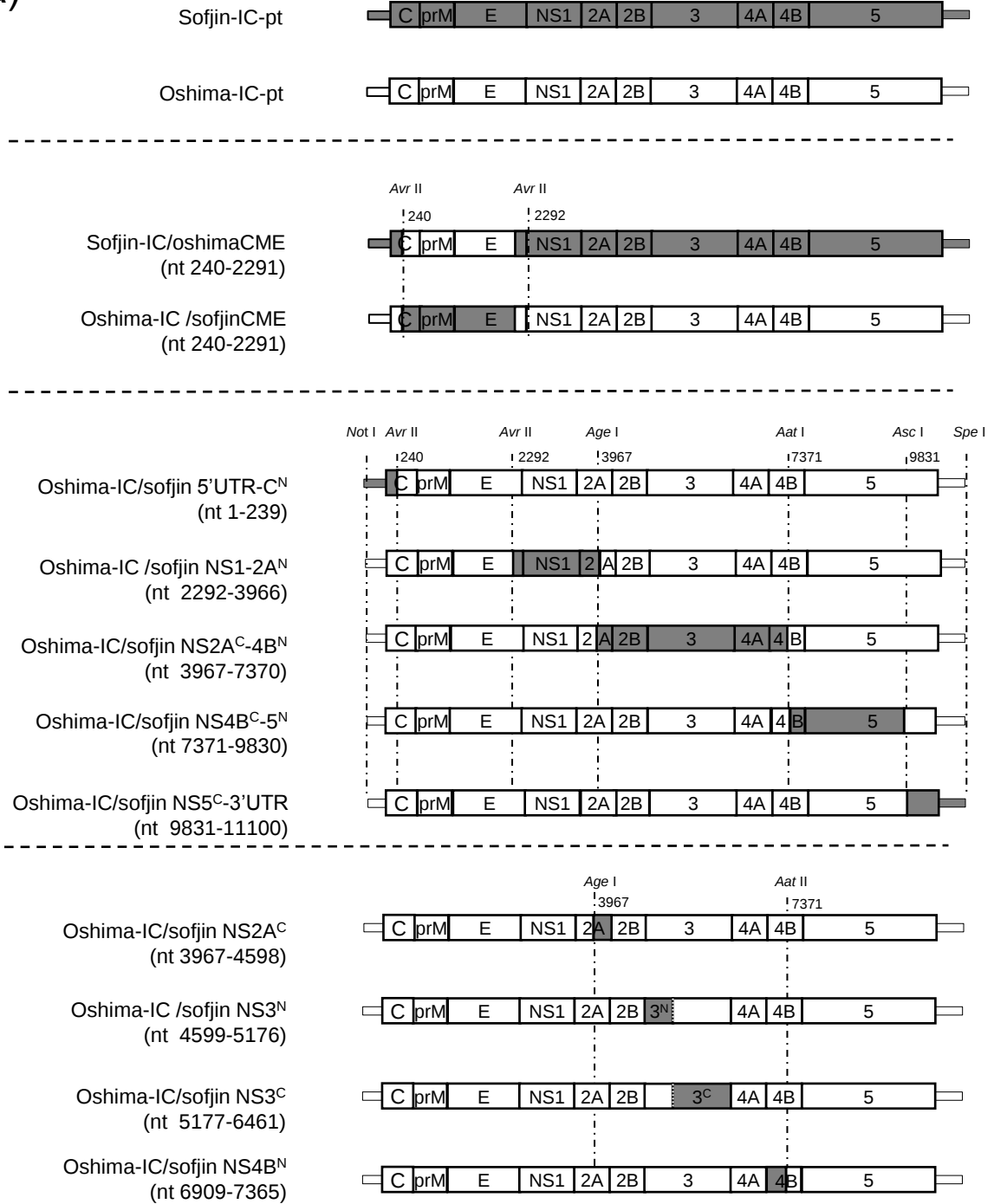
gemone region	Amino acid position	Amino acid differences		replaced regions of chimeric viruses	
		Oshima	Sofjin		
5'-UTR	-	-	-	5'UTR-C ^N	
C	32	Arg	Gln	C ^C -E ^N	
	43	Met	Leu		
prM	64	Asn	Lys		
	171	Val	Leu		
E	152	Val	Ala	NS1-2A ^N	
	171	Val	Ala		
	306	Val	Met		
NS1	462	Met	Val		
	463	Ala	val		
	58	Leu	Val		
	72	Val	Ala		
	93	Phe	Leu		
	138	Ile	Thi		
NS2A	239	Asp	Gly		
	346	Val	Ile		
	52	Arg	Lys		
	168	Ile	Thr		
	169	Ala	Val		
NS2B	225	Val	Ile		
No difference				NS2A ^C -4B ^N	NS 2A ^C
NS3	45	Phe	Ser		
	62	Ser	Tyr		
	63	Val	Ile		
	68	Val	Ala		
	76	Lys	Arg		
	124	Gly	Arg		
	184	Ile	Thr		
	380	Thr	Ala		
NS4A	485	Gly	Ser		
	608	Arg	His		
No difference				NS4B ^C -5 ^N	NS 3 ^C
NS4B	24	Leu	Val		
	198	Ser	Ala		
	227	Ser	Gly		
NS5	15	Lys	Arg		
	101	Arg	Lys		
	130	Met	Val		
	519	Tyr	His		
	634	Thr	Ser		
	641	Phe	Leu		
	677	Arg	Gly		
	692	Val	Ile		
NS5	778	Phe	Leu	NS5 ^C -3'UTR	
	827	Gly	Ser		
	832	Val	Ala		
	862	Arg	Lys		
3'-UTR	-	-	-		

Table 2. Physical differences among mice infected with Sofjin-IC-pt, Oshima-IC-pt or the recombinant viruses^a.

	Onset of disease (days)	Survival time (days)	Morbidity (%)	Mortality (%)
Sofjin-IC-pt	7.7 ± 0.8**	9.0 ± 1.5**	100	100
Oshima-IC-pt	11.9 ± 1.7	18.4 ± 5.3	90	50
Sofjin-IC/oshimaCME	8.8 ± 0.4**	8.4 ± 0.5**	100	100
Oshima-IC/sofjinCME	9.6 ± 1.8	15.0 ± 6.2	100	60
Oshima-IC/sofjin5'-UTR-C ^N	8.7 ± 1.3**	13.5 ± 4.4	100	100
Oshima-IC/sofjin NS1-2A ^N	9.3 ± 1.5**	13.7 ± 3.2	100	90
Oshima-IC/sofjin NS2A ^C -4B ^N	8.4 ± 0.7**	11.0 ± 2.4**	100	90
Oshima/sofjinNS2A ^C	11.5 ± 2.6	16.0	80	20
Oshima/sofjinNS3 ^N	8.2 ± 1.1**	12.9 ± 4.7	100	100
Oshima/sofjinNS3 ^C	9.8 ± 2.0	13.3 ± 1.5	90	30
Oshima/sofjinNS4B ^N	9.5 ± 1.6	18.0 ± 5.7	100	80
Oshima-IC/sofjinNS4B ^C -5 ^N	9.3 ± 0.7**	13.9 ± 2.5	100	70
Oshima-IC/sofjinNS5 ^C -3'-UTR	7.7 ± 0.7**	8.7 ± 1.2**	100	100
Oshima-IC/NS5-778L	10.0 ± 0.8	17.8 ± 5.0	80	40
Oshima-IC/NS5-827S	8.9 ± 1.0**	14.9 ± 4.9	100	80
Oshima-IC/NS5-832A	9.6 ± 1.5*	15.5 ± 4.7	100	60
Oshima-IC/NS5-862K	9.8 ± 1.8*	17.4 ± 5.7	100	50
Oshima-IC/3'-UTR-variable (dSL2/3/4)	8.6 ± 0.5**	10.4 ± 1.6*	100	100
Oshima-IC/3'-UTR-core	9.6 ± 1.8	13.7 ± 4.5	100	60
Oshima_dSL2/3	8.6 ± 0.8**	9.7 ± 0.7**	100	100
Oshima_dSL4	8.2 ± 0.4**	9.7 ± 0.4**	100	100
Oshima_polyA	9.1 ± 0.7**	10.0 ± 1.1**	100	100

^a Five adult mice C57BL/6 were infected with Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME, and ten mice were infected with the others. One asterisk (*) or double asterisk (**) denotes a significant difference between Oshima-IC and the other viruses ($p < 0.05$ or 0.01), respectively.

(a)



(b)

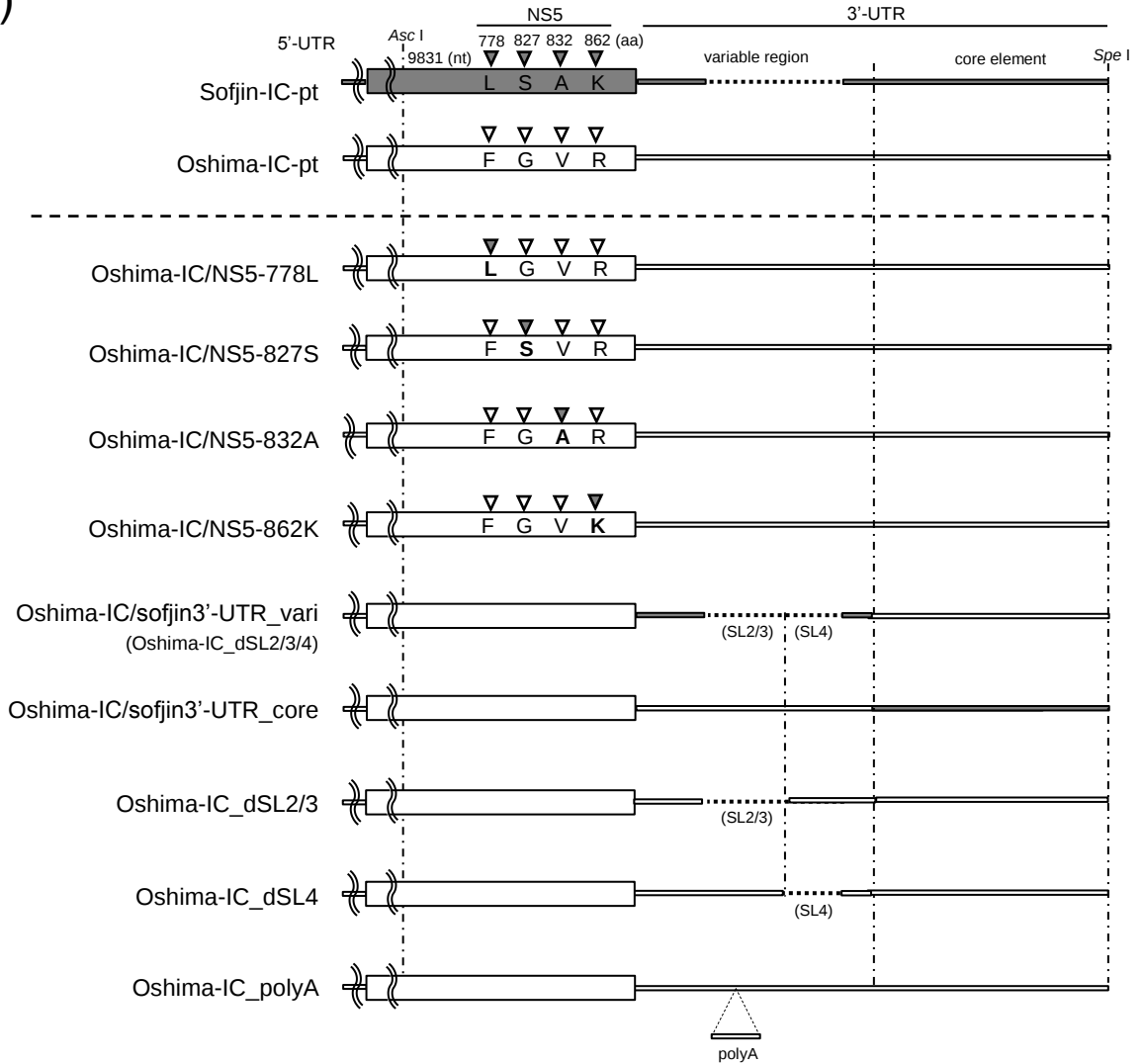
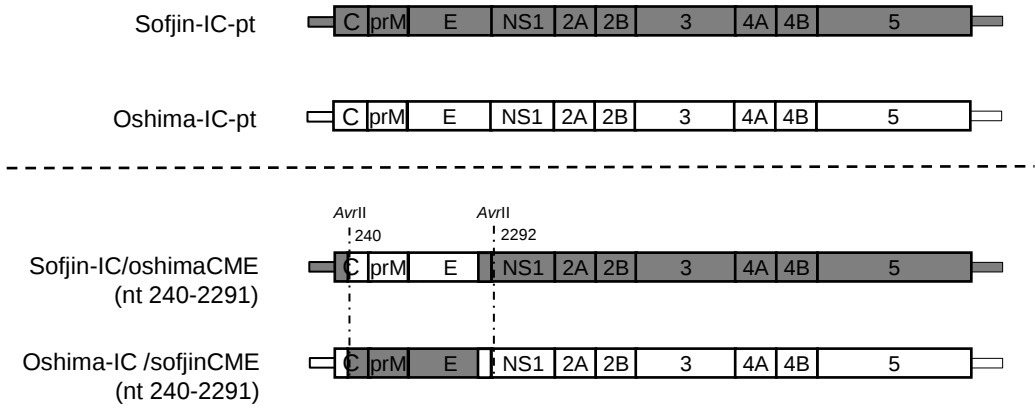


Fig. 1 Recombinant viruses used in this study.

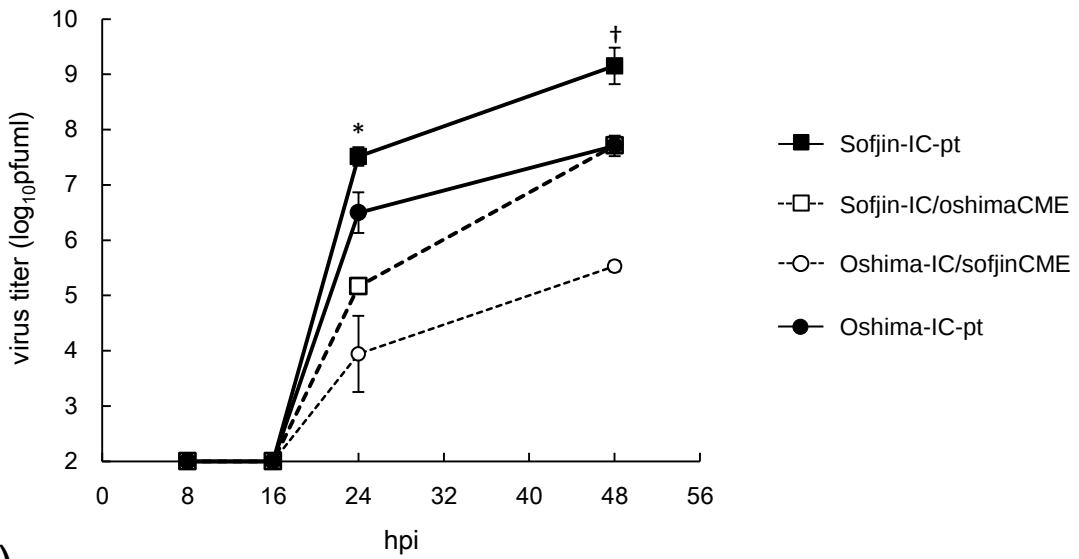
(a) Replacements of the structural and non-structural protein. **(b)** Amino acid substitutions in NS5 and a replacement, deletions or an insertion in the 3'-UTR.

Fig.2

(a)



(b)



(c)

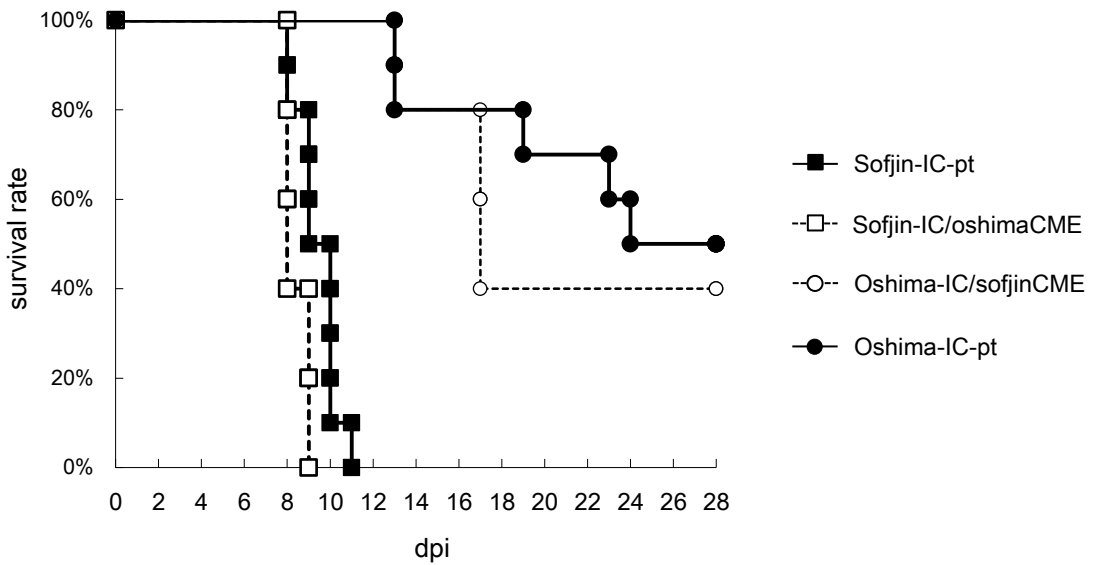


Fig.2 Effect of replacement of the TBEV region encoding most of the structural proteins on pathogenicity in mice. **(a)** Schematic representation of the genomes of recombinant Sofjin-IC and Oshima-IC viruses. Sofjin-IC/OshimaCME and Oshima-IC/sofjinCME were constructed by replacement of the coding region for most structural proteins (nucleotide 240-2291) with that of Oshima-IC-pt and Sofjin-IC-pt, respectively. Sofjin-IC and Oshima-IC regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in mouse neuroblastoma (NA) cells. NA cells were infected with each virus at a MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data are the means $\pm$ SD of three independent experiments. *At 24 hours post-infection (hpi), Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME showed significant differences from Oshima-IC-pt and Sofjin-IC-pt ($p < 0.05$). †At 48 hpi, a significant difference was observed between Sofjin-IC-pt and both chimeric viruses, and Oshima-IC/sofjinCME showed a significant difference from Oshima-IC-pt ($p < 0.05$). **(c)** Survival of mice inoculated with Sofjin-IC, Oshima-IC and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed square), Sofjin-IC/oshimaCME (open square), Oshima-IC/sofjinCME (open circle) and Oshima-IC-pt (closed circle).

Fig.3

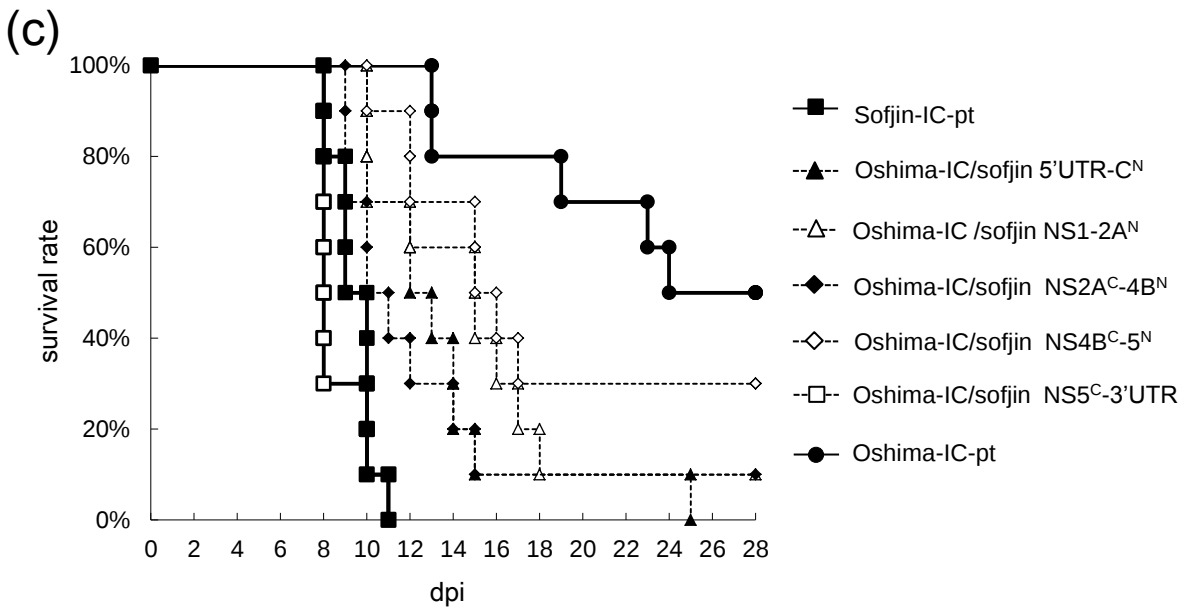
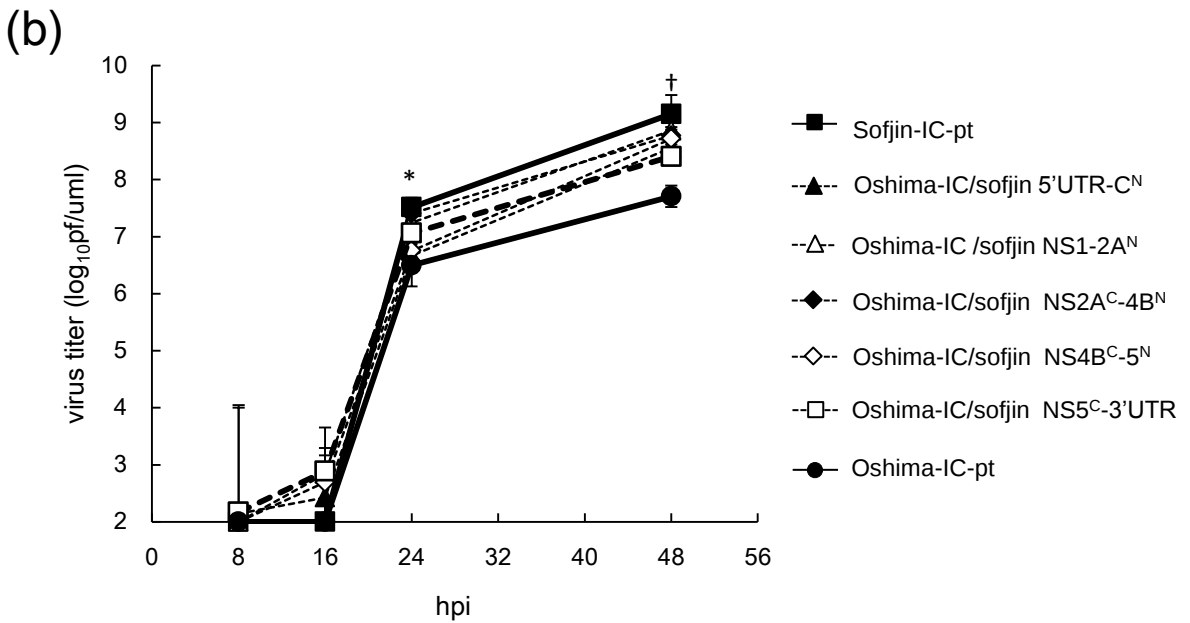
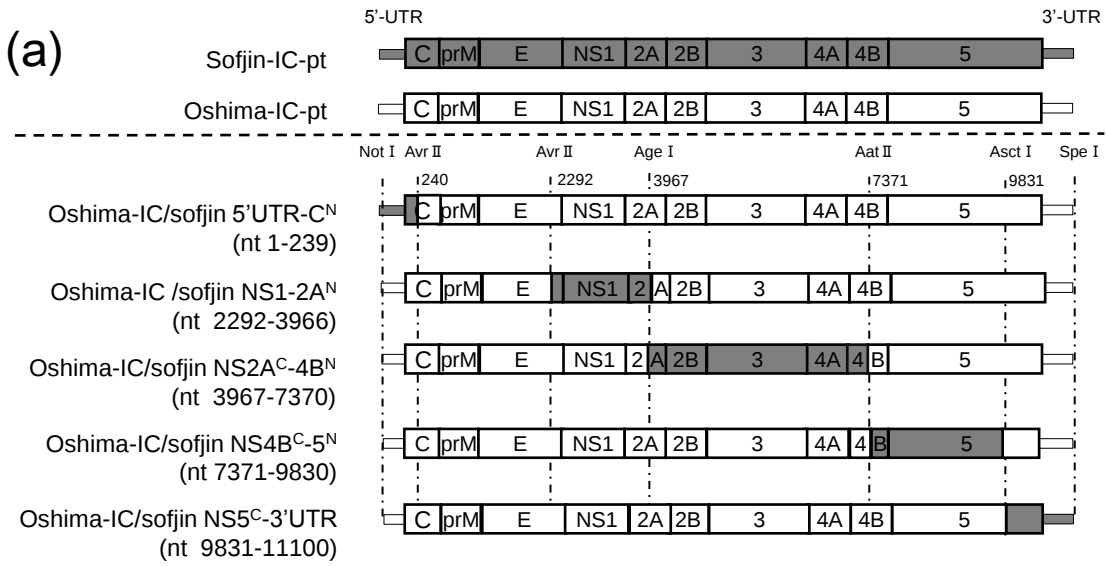


Fig.2 Effect of replacement of TBEV untranslated regions and the region encoding non-structural proteins on the pathogenicity in mice.

(a) Schematic representation of the genomes of the chimeric viruses. Each Oshima-based virus was constructed by replacement of the 5'-UTR and the N-terminus of C (5'UTR-C^N), the C-terminal region of E and NS1 and the N-terminal region of NS2A (NS1-2A^N), the C-terminus of NS2A and NS3 and the N-terminus of NS4B (NS2A^C-4B^N), the C-terminus of NS4B and the N-terminus of NS5 (NS4B^C-5^N), or the C-terminus of NS5 and the 3'-UTR (NS5^C-3'UTR) with the respective region of Sofjin-IC. The Sofjin-IC- and Oshima-IC regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in NA cells. NA cells were infected with each virus at an MOI of 1. Viral titres at each time point were determined in BHK-21 cells. The data are the means $\pm$ SD of three independent experiments. *At 24 hpi, Oshima-IC/sofjinNS1-2A^N, Oshima-IC/sofjin5'UTR-C^N and Oshima-IC/sofjinNS4B^C-5^N showed significant differences from Oshima-IC-pt, and Oshima-IC/sofjinNS5^C-3'UTR and Oshima-IC/sofjinNS4B^C-5^N showed significant differences from Sofjin-IC-pt ($p < 0.05$). †At 48 hpi, a significant difference was observed between Sofjin-IC-pt and Oshima-IC/sofjin5'UTR-C^N or Oshima-IC/sofjinNS5^C-3'UTR, and between Oshima-IC-pt and the other viruses ($p < 0.05$). **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt, and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed square), Oshima-IC/sofjin5'UTR-C^N (closed triangle), Oshima-IC/sofjinNS1-2A^N (open triangle), Oshima-IC/sofjinNS2A^C-4B^N (closed diamond), Oshima-IC/sofjinNS4B^C-5^N (open diamond), Oshima-IC/sofjinNS5^C-3'UTR (open square), or Oshima-IC-pt (closed circle).

Fig.4

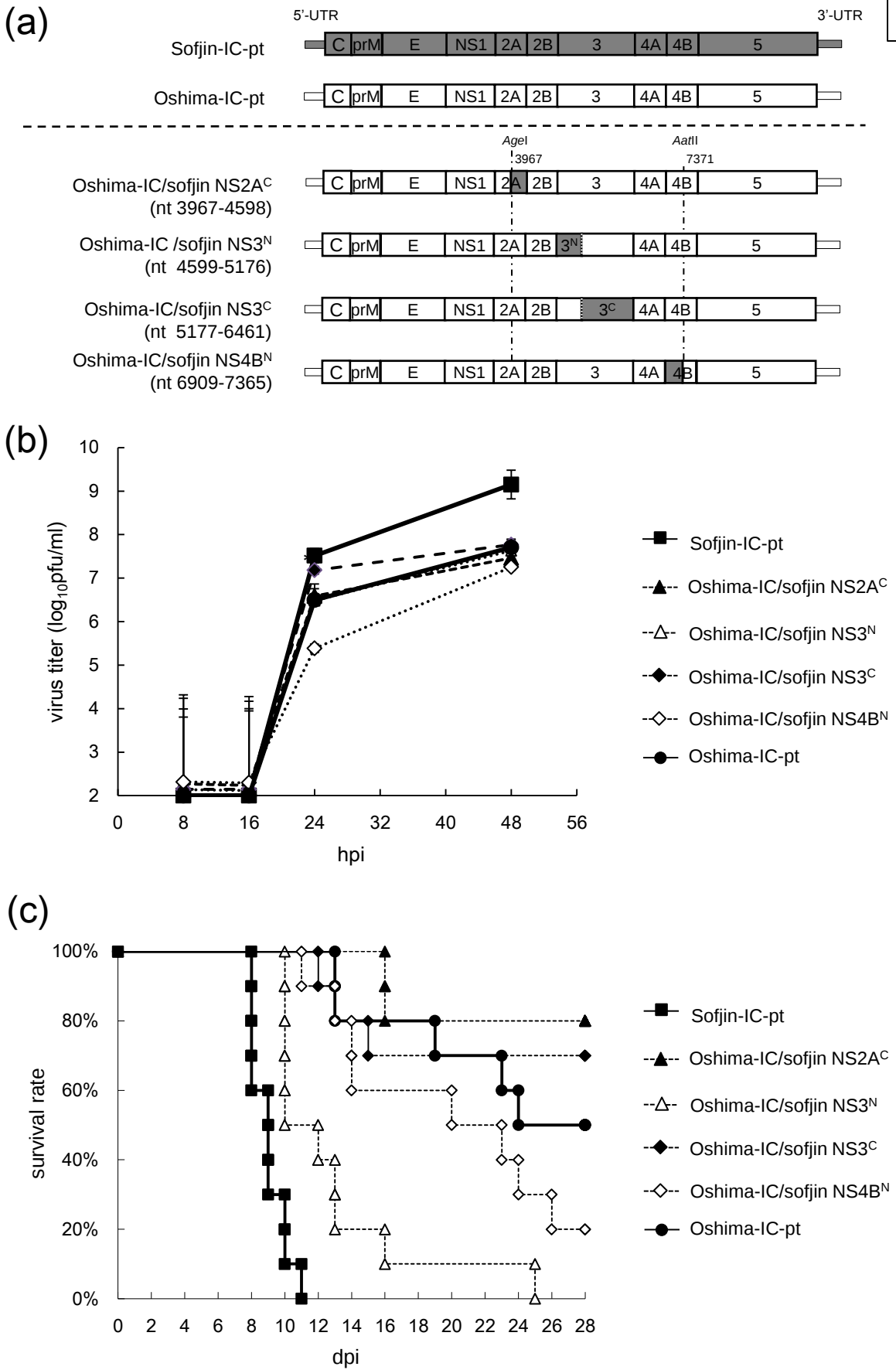
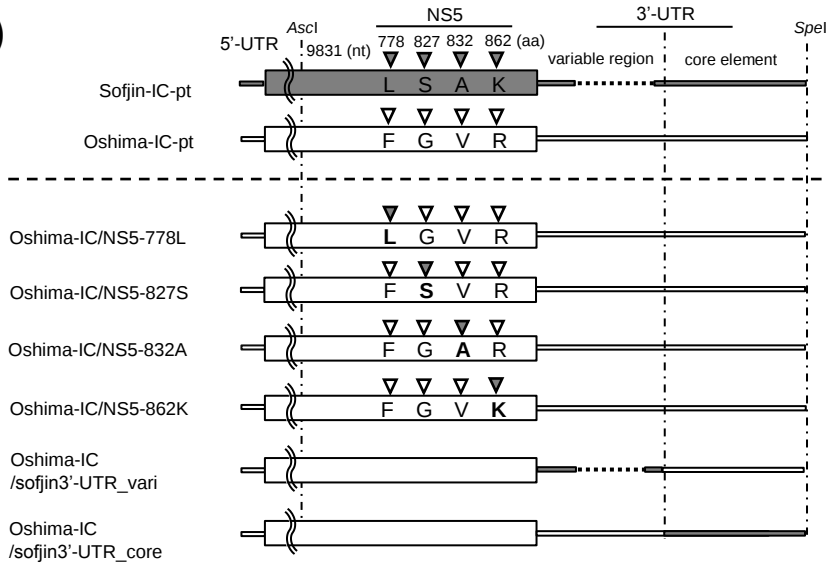


Fig.4 Effect of replacement of the TBEV region encoding non-structural proteins (NS2A, NS3 and NS4B) on the pathogenicity in mice.

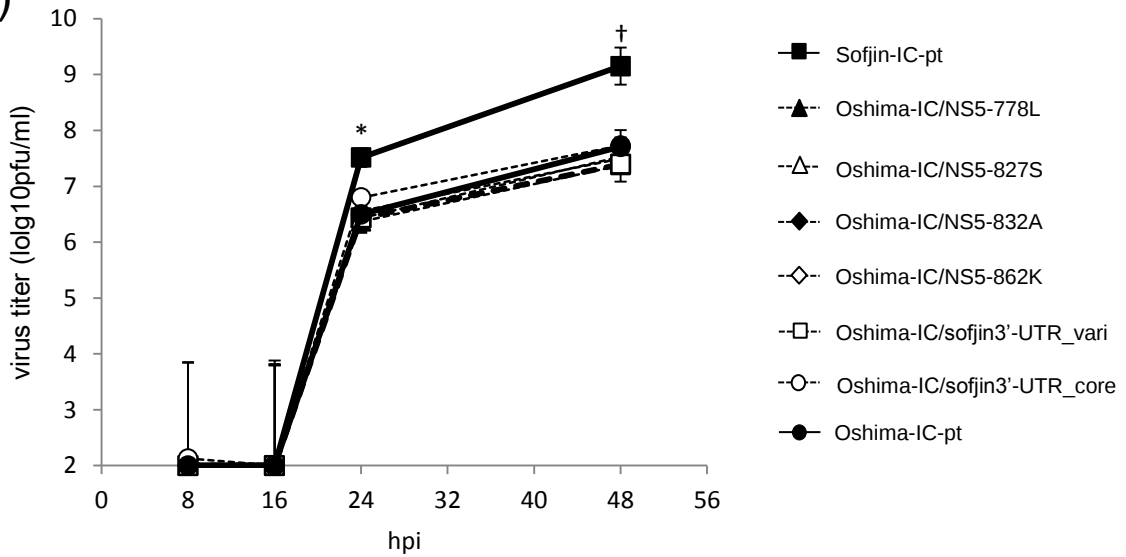
(a) Schematic representation of the genomes of the chimeric viruses. Each Oshima-based virus was constructed by replacement of the NS2A, the N- and C-terminal region of NS3 and the C-terminal region of NS4B with each of Sofjin-IC-pt. The Sofjin-IC-pt and Oshima-IC-pt regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in NA cells. NA cells were infected with each virus at a MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data are the means $\pm$ SD of three independent experiments. *At 24 hpi., Oshima-IC/sofjinNS3^C and Oshima-IC/sofjinNS4B^N showed significant differences from Oshima-IC-pt, and Oshima-IC/sofjinNS2A^C, Oshima-IC/sofjinNS3^N and Oshima-IC/sofjinNS4B^N showed significant differences from Sofjin-IC-pt ($p < 0.05$). †At 48 hpi., significant differences were observed between Sofjin-IC-pt and the other viruses ($p < 0.05$). No significant differences between Oshima-IC-pt and each chimeric virus were observed. **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu Sofjin-IC-pt (closed square), Oshima-IC/sofjinNS2A^C (closed triangle), Oshima-IC /sofjinNS3^N (open triangle), Oshima-IC/sofjinNS3^C (closed diamond), Oshima-IC/sofjinNS4B^N (open diamond), or Oshima-IC-pt (closed circle).

Fig.5

(a)



(b)



(c)

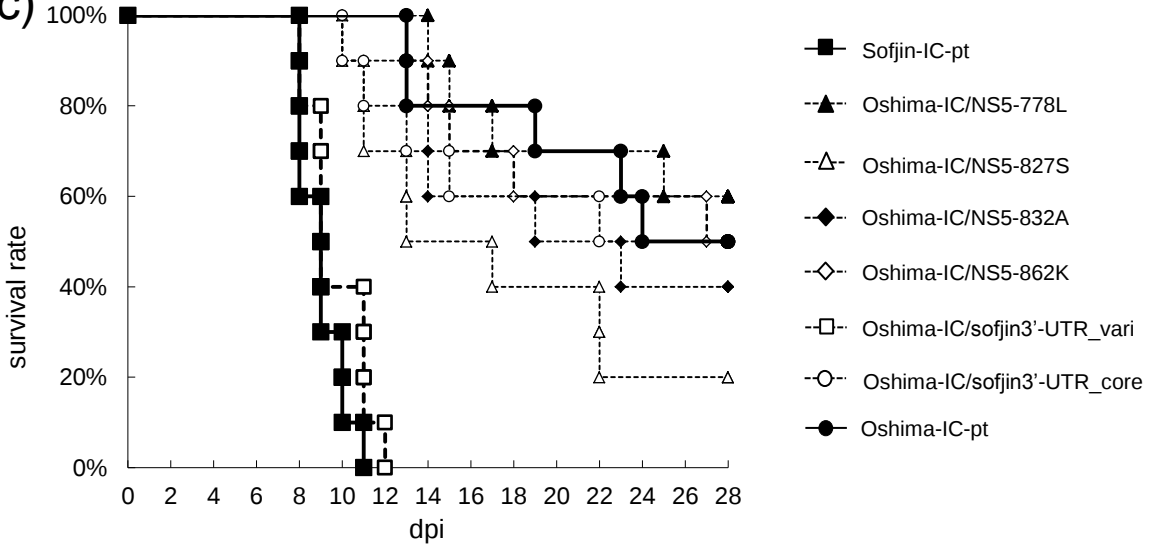


Fig.5 Effect of substitutions of TBEV amino acids in NS5 and replacement of the 3'-UTR.

(a) Schematic representation of the genome of recombinant viruses. Single-amino acid substitutions were introduced at NS5 positions 778 (NS5-778L), 827 (NS5-827S), 832 (NS5-832A), and 862 (NS5-862K) of Oshima-IC. The gray and white arrowheads indicate amino acids derived from Sofjin-IC-pt and Oshima-IC-pt, respectively. Oshima-IC/sofjin3'-UTR_vari and Oshima-IC/sofjin3'-UTR_core are Oshima-IC chimeric viruses in which the variable region and core element of the 3'-UTR were replaced with those of Sofjin-IC-pt. The gray lines indicate the regions derived from the 3'-UTR of Sofjin-IC-pt. The broken line indicates the region lacking in Sofjin-IC-pt. **(b)** Growth curves of each virus in NA cells. NA cells were infected with each virus at a MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data are the means $\pm$ SD of three independent experiments. *At 24 hpi, the chimeric viruses except for Oshima-IC/sofjin-3'UTR_core showed significant differences from Sofjin-IC-pt ($p < 0.05$). †At 48 hpi, significant differences were observed between Sofjin-IC-pt and the other viruses ($p < 0.01$). No significant difference between Oshima-IC-pt and each chimeric virus was observed at 24 and 48 hpi. **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed square), Oshima-IC/NS5-778L (closed triangle), Oshima-IC/NS5-827S (open triangle), Oshima-IC/NS5-832A (closed diamond), Oshima-IC/NS5-862K (open diamond), Oshima-IC/sofjin3'-UTR_vari (open square), Oshima-IC/sofjin3'-UTR_core (open circle), and Oshima-IC-pt (closed circle).

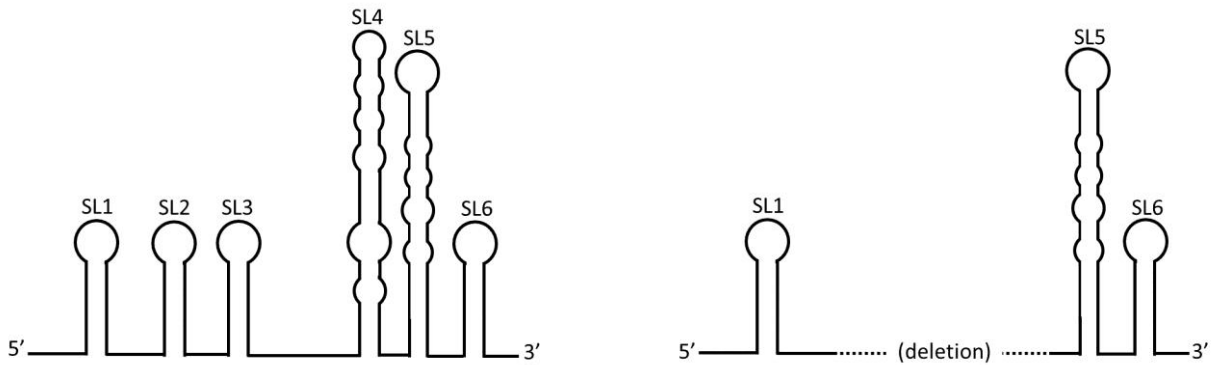
Oshima-IC-ptSofjin-IC-pt

Fig.6 The RNA secondary structures of the variable region (10377-10757 nucleotide of Oshima and 10377-10550 nucleotide of Sofjin) were predicted by RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). Six stem loop (SL) structures (SL1-6) were observed in the variable region of Oshima-IC-pt and 3 SL (SL1, SL5 and SL6) were observed in Sofjin-IC-pt.

Fig.7

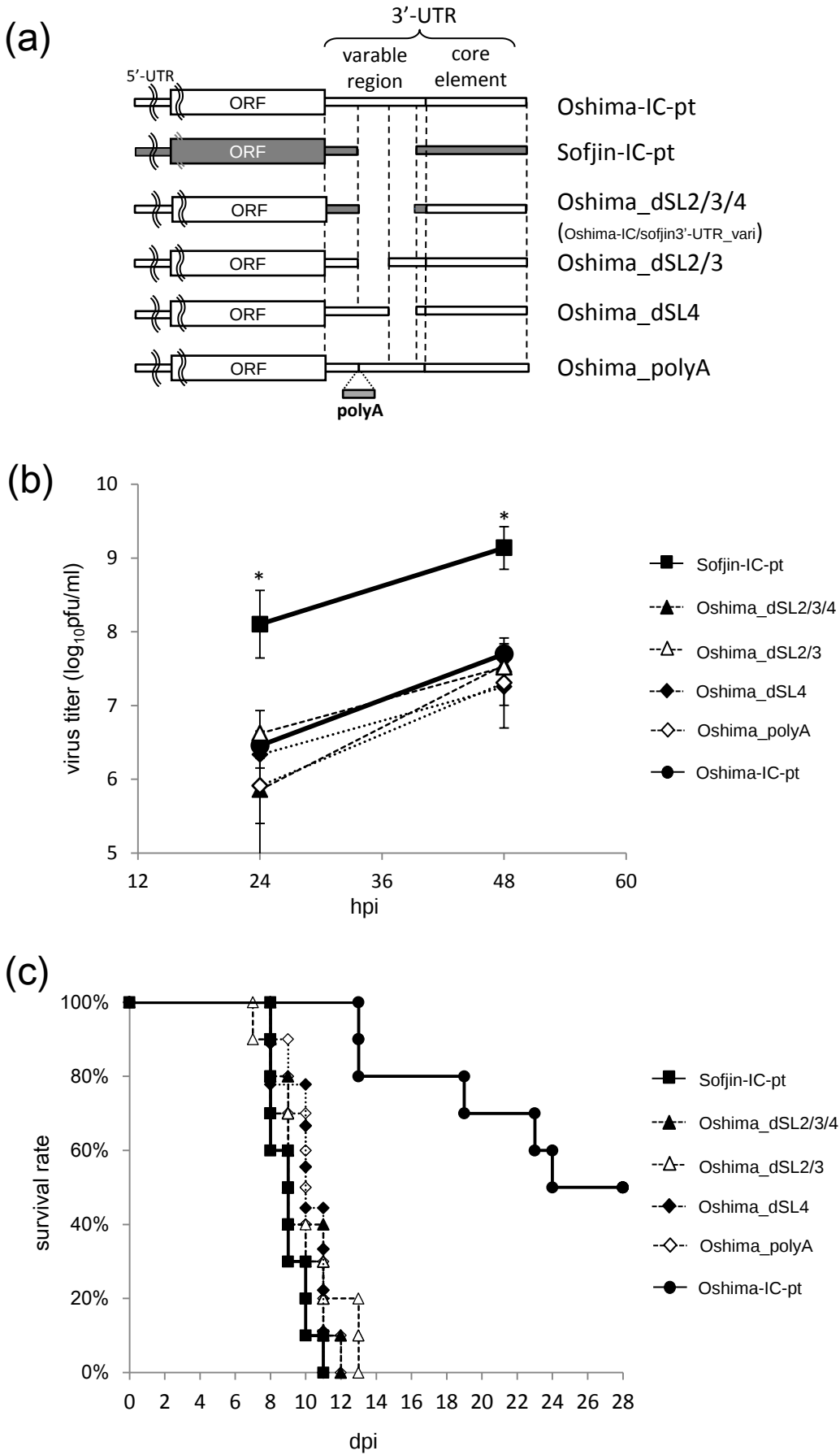


Fig.7 Effect of deletion and insertion of polyA sequence in the variable region.

(a) Schematic representation of the genome of recombinant viruses. Oshima-IC/sofjin_vari was used as a mutant virus with deletion of the region including SL2-4; Oshima_dSL2/3/4. Oshima_dSL2/3 and Oshima_dSL4 have a deletion of the region of SL2-3 or SL4, respectively. Oshima_polyA has insertion of 35 adenine (polyA) in the position of deletion in the variable region of Sofjin. **(b)** Growth curve of each virus in NA cells. NA cells were infected with each virus at a MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data are the means $\pm$ SD of three independent experiments. *At 24 hpi and 48 hpi, significant differences were observed between Sofjin-IC-pt and the other viruses ($p < 0.05$). No significant difference between Oshima-IC-pt and each mutant virus was observed. **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the mutant viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed square), Oshima_dSL2/3/4 (closed triangle), Oshima_dSL2/3 (open triangle), Oshima_dSL4 (closed diamond), Oshima_polyA (open diamond) and Oshima-IC-pt (closed circle).

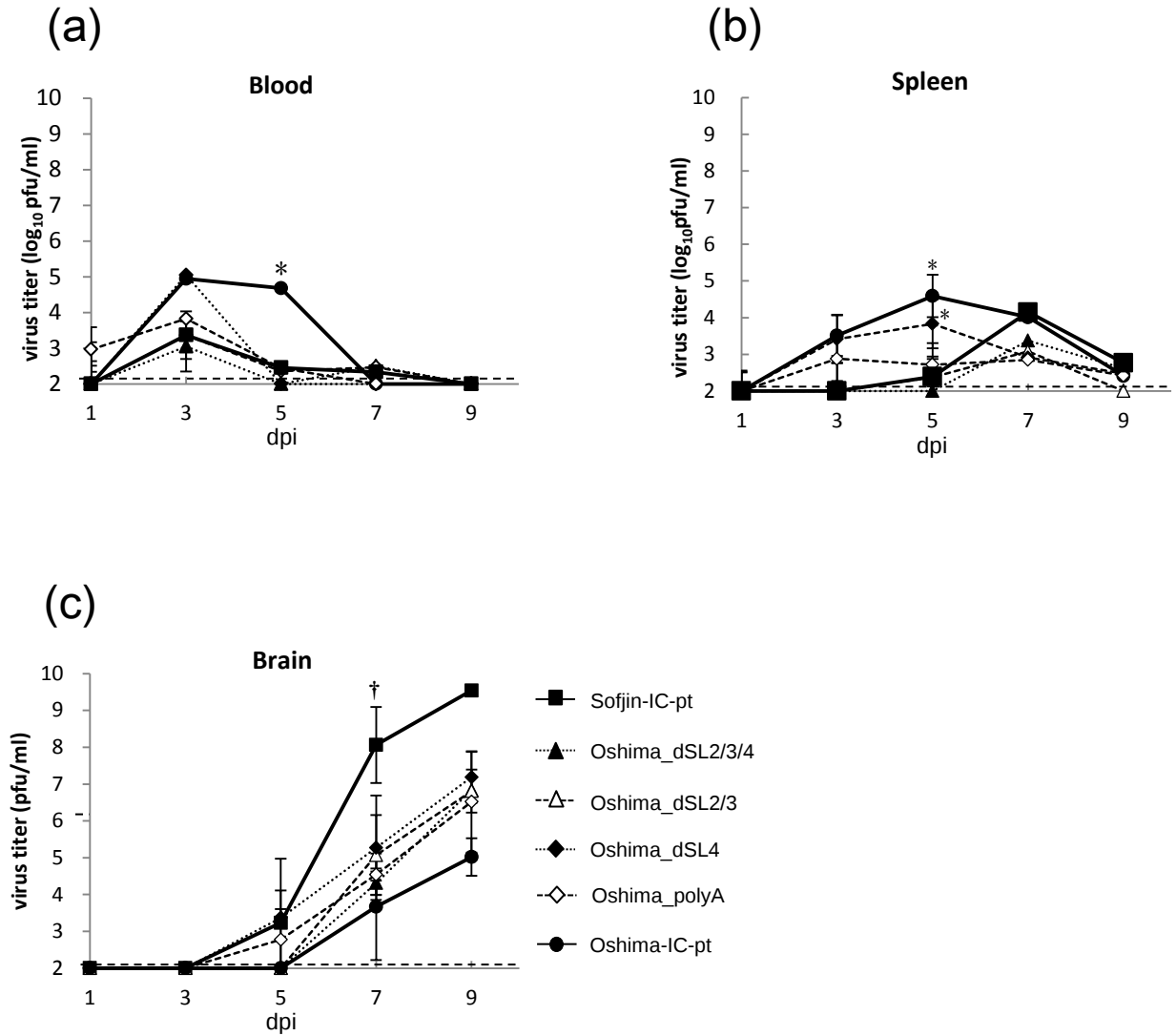


Fig. 8 Effects of the replacement of the variable region on viral multiplication in organs.

Mice were infected with 1,000 pfu of Sofjin-IC-pt, Oshima_dSL2/3/4, Oshima_dSL2/3, Oshima_dSL4, Oshima_polyA and Oshima-IC-pt. Virus titers in the blood (a), spleen (b), and brain (c) at the indicated days after infection were determined by plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (100 pfu/mL). Error bars represent the SD (n=3). At 9 days p.i., number of mice infected with Sofjin-IC-pt or Oshima_polyA was 2 because of the death before the time point. An asterisk (*) or dagger (†) denotes a significant difference compared with or Sofjin-pt-IC and Oshima-IC-pt, respectively ($p < 0.05$).

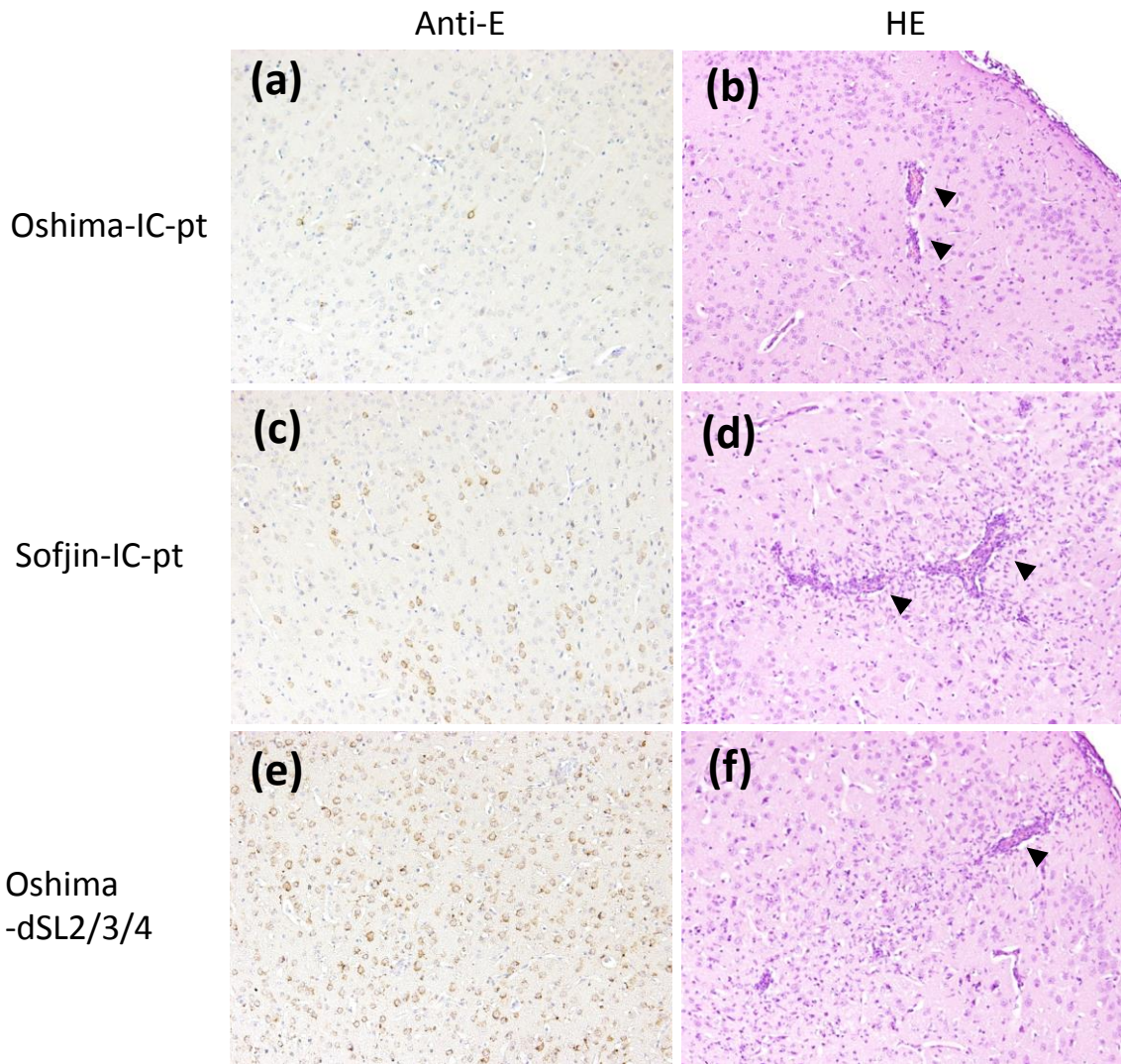


Fig. 9 Histopathological features in the brain of mice at 9 days after subcutaneous infection.

Mice were infected with 10^3 pfu of Oshima-IC-pt ((a), (b)) or Sofjin-IC-pt ((c), (d)) and Oshima-dSL2/3/4 ((e), (f)). TBEV antigens were detected using E-protein-specific antibodies (brown signal in left columns). Non-suppurative encephalitis including perivascular cuffing (arrowhead) was observed in mice infected with each virus (right columns).

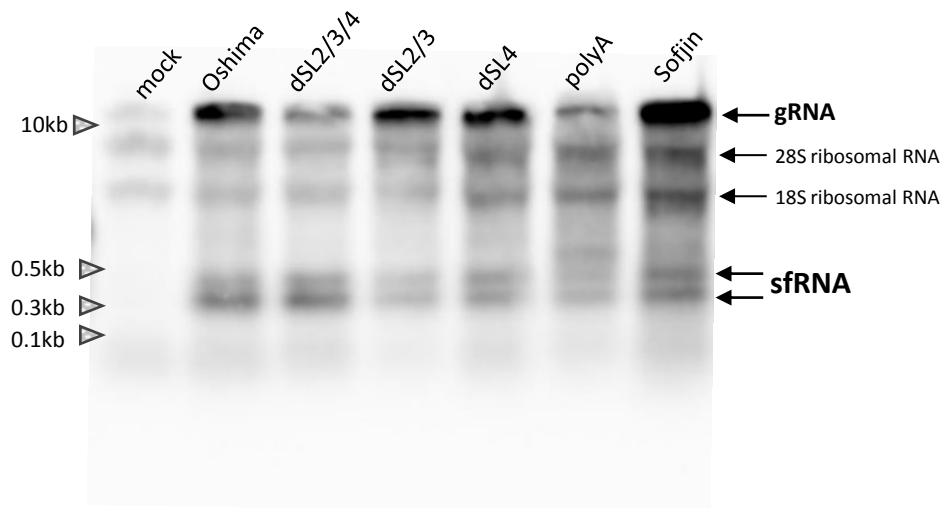
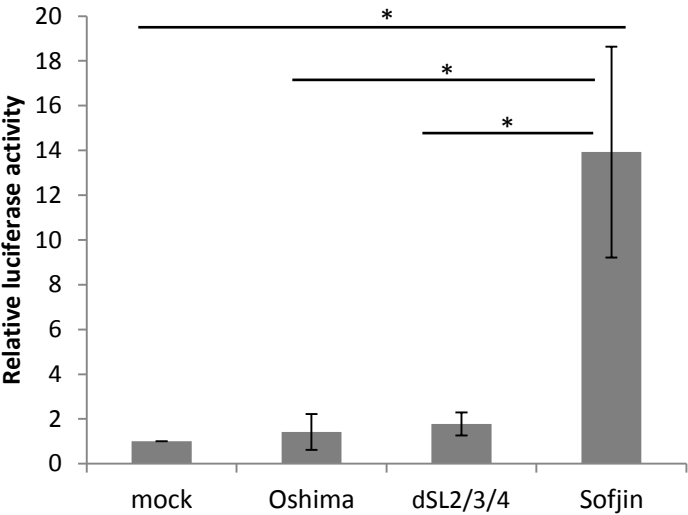


Fig. 10 TBEV produced subgenomic flavivirus RNA (sfRNA).

BHK-21 cells were infected with each virus and harvested at 48 hpi. The cells were lysed and RNA was extracted. The total RNA (4 μ g) were loaded into each well and genomic RNA (gRNA) and sfRNA were detected by Northern blotting, using a DIG-labeled probe to detect the 3'-UTR.

(a) Activity of IFN-β promoter in infected SYM-1 cells



(b) Activity of ISRE promoter in infected NA cells

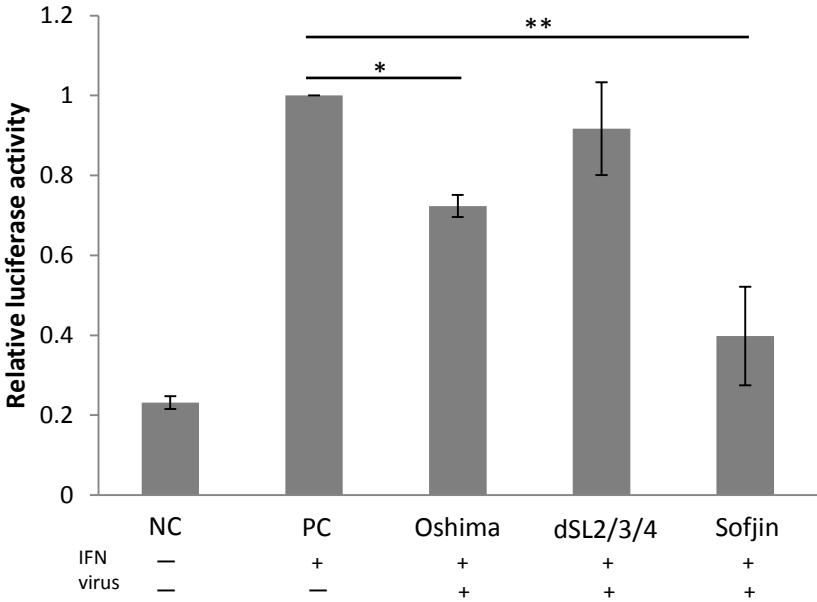


Fig.11 The deletion in the variable region did not affect IFN-mediated antiviral response.

SYM-1 and NA cells were transfected with 1 µg of pISRE-Luc Cis-Reporter Plasmid or 4 × IRF-3-Luc reporter plasmid, respectively. At 24 hpi, the cells were inoculated with each viruses (NC: negative control). To activate ISRE promoter, NA cells were treated with 500 U/ml mouse IFN-α at 24 hpi (PC: positive control), and three hours after the treatment, the NA cells were harvested. The SYM-1 cells were collected at 48 hpi. The cells were lysed and the luciferase activities were measured. The data are presented as means ($\pm$ the SD) of three independent replicates. Asterisk (*) and (**) denote a significant difference ($p < 0.05$ or 0.01).

SUMMARY

Tick-borne encephalitis virus (TBEV) is maintained among ticks and mammals in nature, and human can be infected with tick bite. Although about 10,000 cases have been reported annually in European countries and Russia, there is no attenuated live vaccine and anti-viral therapy. The virulence of TBEV varies among the strains. However, the mechanism of the viral pathogenicity is unknown. To clarify the mechanism, I tried to identify the virulence factors by reverse genetics, using two different virulence viruses of the Far-Eastern subtype of TBEV; a highly pathogenic strain Sofjin-HO and a low pathogenic strain Oshima 5-10. I found that the variable region of the 3'-UTR is a critical virulence factor and the deletion in the variable region affected the different virulence in mice. Partial deletions or addition of polyA sequence in this region of Oshima also increased the virulence to the same level with Sofjin, although they did not affect the viral multiplication in mice brain and cultured cells. These mutations did not change the production of subgenomic flavivirus RNA from the 3'-UTR, and the induction of interferon (IFN) and IFN-stimulated genes. These data suggested that the whole conformational structure of the variable region is associated with the pathogenicity of the Far-Eastern subtype of TBEV by unknown mechanisms. These findings encourage further research to identify the pathogenic mechanisms of TBEV and develop prevention and therapeutic strategies for TBE.

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REFERENCES

1. Kaiser, R., P. Kimmig, and J. Suss, *[Re-evaluation of the risk areas for tick-borne encephalitis in Germany]*. MMW Fortschr Med, 2007. **149**(5): p. 34-5.
2. Lindquist, L. and O. Vapalahti, *Tick-borne encephalitis*. Lancet, 2008. **371**(9627): p. 1861-71.
3. Mansfield, K.L., et al., *Tick-borne encephalitis virus - a review of an emerging zoonosis*. J Gen Virol, 2009. **90**(Pt 8): p. 1781-94.
4. Gritsun, T.S., V.A. Lashkevich, and E.A. Gould, *Tick-borne encephalitis*. Antiviral Res, 2003. **57**(1-2): p. 129-46.
5. Heinz, F.X. and C. Kunz, *Tick-borne encephalitis and the impact of vaccination*. Arch Virol Suppl, 2004(18): p. 201-5.
6. Grard, G., et al., *Genetic characterization of tick-borne flaviviruses: new insights into evolution, pathogenetic determinants and taxonomy*. Virology, 2007. **361**(1): p. 80-92.
7. Heinz, F.X. and S.L. Allison, *Flavivirus structure and membrane fusion*. Adv Virus Res, 2003. **59**: p. 63-97.
8. Chambers, T.J., et al., *Flavivirus genome organization, expression, and replication*. Annu Rev Microbiol, 1990. **44**: p. 649-88.
9. Kofler, R.M., et al., *Spontaneous mutations restore the viability of tick-borne encephalitis virus mutants with large deletions in protein C*. J Virol, 2003. **77**(1): p. 443-51.
10. Kofler, R.M., F.X. Heinz, and C.W. Mandl, *Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence*. J Virol, 2002. **76**(7): p. 3534-43.
11. Lobigs, M. and A. Mullbacher, *Recognition of vaccinia virus-encoded major histocompatibility complex class I antigens by virus immune cytotoxic T cells is independent of the polymorphism of the peptide transporters*. Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2676-80.
12. Navarro-Sanchez, E., et al., *Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses*. EMBO Rep, 2003. **4**(7): p. 723-8.
13. Kopecky, J., et al., *A putative host cell receptor for tick-borne encephalitis virus identified by anti-idiotypic antibodies and virus affinoblotting*. Intervirology, 1999. **42**(1): p. 9-16.

14. Kozlovskaya, L.I., et al., *GAG-binding variants of tick-borne encephalitis virus*. Virology, 2010. **398**(2): p. 262-72.
15. Bazan, J.F. and R.J. Fletterick, *Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses*. Virology, 1989. **171**(2): p. 637-9.
16. Fischl, W., et al., *Changing the protease specificity for activation of a flavivirus, tick-borne encephalitis virus*. J Virol, 2008. **82**(17): p. 8272-82.
17. Matusan, A.E., et al., *Mutagenesis of the Dengue virus type 2 NS3 protein within and outside helicase motifs: effects on enzyme activity and virus replication*. J Virol, 2001. **75**(20): p. 9633-43.
18. Egloff, M.P., et al., *An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization*. EMBO J, 2002. **21**(11): p. 2757-68.
19. Park, G.S., et al., *Identification of residues critical for the interferon antagonist function of Langat virus NS5 reveals a role for the RNA-dependent RNA polymerase domain*. J Virol, 2007. **81**(13): p. 6936-46.
20. Khromykh, A.A., et al., *Essential role of cyclization sequences in flavivirus RNA replication*. J Virol, 2001. **75**(14): p. 6719-28.
21. Kofler, R.M., et al., *Functional analysis of the tick-borne encephalitis virus cyclization elements indicates major differences between mosquito-borne and tick-borne flaviviruses*. J Virol, 2006. **80**(8): p. 4099-113.
22. Mandl, C.W., et al., *Spontaneous and engineered deletions in the 3' noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus*. J Virol, 1998. **72**(3): p. 2132-40.
23. Mandl, C.W., C. Kunz, and F.X. Heinz, *Presence of poly(A) in a flavivirus: significant differences between the 3' noncoding regions of the genomic RNAs of tick-borne encephalitis virus strains*. J Virol, 1991. **65**(8): p. 4070-7.
24. Bredenbeek, P.J., et al., *A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication*. J Gen Virol, 2003. **84**(Pt 5): p. 1261-8.
25. Leonova, G.N., et al., *Comprehensive assessment of the genetics and virulence of tick-borne encephalitis virus strains isolated from patients with inapparent and clinical forms of the infection in the Russian Far East*. Virology, 2013. **443**(1): p. 89-98.
26. Formanova, P., et al., *Full genome sequences and molecular characterization of tick-borne encephalitis virus strains isolated from human patients*. Ticks Tick Borne Dis, 2015. **6**(1): p. 38-46.

27. Pijlman, G.P., et al., *A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity*. Cell Host Microbe, 2008. **4**(6): p. 579-91.
28. Chang, R.Y., et al., *Japanese encephalitis virus non-coding RNA inhibits activation of interferon by blocking nuclear translocation of interferon regulatory factor 3*. Vet Microbiol, 2013. **166**(1-2): p. 11-21.
29. Detje, C.N., et al., *Local type I IFN receptor signaling protects against virus spread within the central nervous system*. J Immunol, 2009. **182**(4): p. 2297-304.
30. Muller, U., et al., *Functional role of type I and type II interferons in antiviral defense*. Science, 1994. **264**(5167): p. 1918-21.
31. Diamond, M.S., et al., *Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons*. J Virol, 2000. **74**(11): p. 4957-66.
32. Lin, R.J., et al., *Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection*. J Virol, 2004. **78**(17): p. 9285-94.
33. Perelygin, A.A., et al., *Positional cloning of the murine flavivirus resistance gene*. Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9322-7.
34. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
35. Yoneyama, M. and T. Fujita, *[Virus-induced expression of type I interferon genes]*. Uirusu, 2004. **54**(2): p. 161-7.
36. Weber, E., et al., *Type I interferon protects mice from fatal neurotropic infection with Langat virus by systemic and local antiviral responses*. J Virol, 2014. **88**(21): p. 12202-12.
37. Best, S.M., et al., *Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist*. J Virol, 2005. **79**(20): p. 12828-39.
38. Ecker, M., et al., *Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia*. J Gen Virol, 1999. **80** (Pt 1): p. 179-85.
39. Dumpis, U., D. Crook, and J. Oksi, *Tick-borne encephalitis*. Clin Infect Dis, 1999. **28**(4): p. 882-90.
40. Barkhash, A.V., et al., *Variability in the 2'-5'-oligoadenylate synthetase gene cluster is associated with human predisposition to tick-borne encephalitis virus-induced disease*. J Infect Dis, 2010. **202**(12): p. 1813-8.
41. Goto, A., et al., *Genetic and biological comparison of tick-borne encephalitis viruses from Hokkaido and far-eastern Russia*. Jpn J Vet Res, 2002. **49**(4): p. 297-307.
42. Chiba, N., et al., *Protection against tick-borne encephalitis virus isolated in Japan*

- by active and passive immunization. *Vaccine*, 1999. **17**(11-12): p. 1532-9.
43. Shi, P.Y., et al., *Infectious cDNA clone of the epidemic west nile virus from New York City*. *J Virol*, 2002. **76**(12): p. 5847-56.
 44. Puri, B., et al., *Construction of a full length infectious clone for dengue-1 virus Western Pacific, 74 strain*. *Virus Genes*, 2000. **20**(1): p. 57-63.
 45. Yun, S.I., et al., *Development and application of a reverse genetics system for Japanese encephalitis virus*. *J Virol*, 2003. **77**(11): p. 6450-65.
 46. Yoshii, K., et al., *Construction of an infectious cDNA clone for Omsk hemorrhagic fever virus, and characterization of mutations in NS2A and NS5*. *Virus Res*, 2011. **155**(1): p. 61-8.
 47. Mandl, C.W., et al., *Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr*. *J Gen Virol*, 1997. **78 (Pt 5)**: p. 1049-57.
 48. Hayasaka, D., et al., *Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of tick-borne encephalitis virus*. *J Gen Virol*, 2004. **85**(Pt 4): p. 1007-18.
 49. Hayasaka, D., et al., *Sub-genomic replicons of Tick-borne encephalitis virus*. *Arch Virol*, 2004. **149**(6): p. 1245-56.
 50. Takano, A., et al., *Construction of a replicon and an infectious cDNA clone of the Sofjin strain of the Far-Eastern subtype of tick-borne encephalitis virus*. *Arch Virol*, 2011. **156**(11): p. 1931-41.
 51. Gritsun, T.S. and E.A. Gould, *Infectious transcripts of tick-borne encephalitis virus, generated in days by RT-PCR*. *Virology*, 1995. **214**(2): p. 611-8.
 52. Sunden, Y., et al., *Intracerebral vaccination suppresses the spread of rabies virus in the mouse brain*. *Microbes Infect*, 2010. **12**(14-15): p. 1163-9.
 53. Yoshii, K., et al., *Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion*. *J Gen Virol*, 2004. **85**(Pt 10): p. 3049-58.
 54. Mandl, C.W., *Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis*. *Virus Res*, 2005. **111**(2): p. 161-74.
 55. Goto, A., et al., *A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness*. *Vaccine*, 2003. **21**(25-26): p. 4043-51.
 56. Mandl, C.W., et al., *Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo*. *J Virol*, 2001. **75**(12): p. 5627-37.
 57. Rumyantsev, A.A., B.R. Murphy, and A.G. Pletnev, *A tick-borne Langat virus mutant*

- that is temperature sensitive and host range restricted in neuroblastoma cells and lacks neuroinvasiveness for immunodeficient mice.* J Virol, 2006. **80**(3): p. 1427-39.
58. Lescar, J., et al., *Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from Dengue virus as a target.* Antiviral Res, 2008. **80**(2): p. 94-101.
 59. Chambers, T.J., et al., *Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein.* Proc Natl Acad Sci U S A, 1990. **87**(22): p. 8898-902.
 60. Gorbalenya, A.E., et al., *N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases.* Nucleic Acids Res, 1989. **17**(10): p. 3889-97.
 61. Potapova, U.V., et al., *NS2B/NS3 protease: allosteric effect of mutations associated with the pathogenicity of tick-borne encephalitis virus.* J Biomol Struct Dyn, 2012. **30**(6): p. 638-51.
 62. Ruzek, D., et al., *Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a Western European field strain of tick-borne encephalitis virus.* Virology, 2008. **374**(2): p. 249-55.
 63. Gritsun, T.S., et al., *Complete sequence of two tick-borne flaviviruses isolated from Siberia and the UK: analysis and significance of the 5' and 3'-UTRs.* Virus Res, 1997. **49**(1): p. 27-39.
 64. Wallner, G., et al., *The flavivirus 3'-noncoding region: extensive size heterogeneity independent of evolutionary relationships among strains of tick-borne encephalitis virus.* Virology, 1995. **213**(1): p. 169-78.
 65. Lei, Y., et al., *Functional interaction between cellular p100 and the dengue virus 3' UTR.* J Gen Virol, 2011. **92**(Pt 4): p. 796-806.
 66. Chien, H.L., C.L. Liao, and Y.L. Lin, *FUSE binding protein 1 interacts with untranslated regions of Japanese encephalitis virus RNA and negatively regulates viral replication.* J Virol, 2011. **85**(10): p. 4698-706.
 67. Ta, M. and S. Vрати, *Mov34 protein from mouse brain interacts with the 3' noncoding region of Japanese encephalitis virus.* J Virol, 2000. **74**(11): p. 5108-15.
 68. De Nova-Ocampo, M., N. Villegas-Sepulveda, and R.M. del Angel, *Translation elongation factor-1alpha, La, and PTB interact with the 3' untranslated region of dengue 4 virus RNA.* Virology, 2002. **295**(2): p. 337-47.
 69. Vashist, S., et al., *La protein binds the predicted loop structures in the 3' non-coding region of Japanese encephalitis virus genome: role in virus replication.* J Gen Virol, 2009. **90**(Pt 6): p. 1343-52.

70. Vashist, S., D. Bhullar, and S. Vratil, *La protein can simultaneously bind to both 3'- and 5'-noncoding regions of Japanese encephalitis virus genome*. DNA Cell Biol, 2011. **30**(6): p. 339-46.
71. Yocupicio-Monroy, M., et al., *Mosquito La protein binds to the 3' untranslated region of the positive and negative polarity dengue virus RNAs and relocates to the cytoplasm of infected cells*. Virology, 2007. **357**(1): p. 29-40.
72. Garcia-Montalvo, B.M., F. Medina, and R.M. del Angel, *La protein binds to NS5 and NS3 and to the 5' and 3' ends of Dengue 4 virus RNA*. Virus Res, 2004. **102**(2): p. 141-50.
73. Ruzek, D., et al., *Breakdown of the blood-brain barrier during tick-borne encephalitis in mice is not dependent on CD8+ T-cells*. PLoS One, 2011. **6**(5): p. e20472.
74. Schnettler, E., et al., *Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and Mammalian cells*. J Virol, 2012. **86**(24): p. 13486-500.
75. Muller, M., H. Ibelgaufts, and I.M. Kerr, *Interferon response pathways--a paradigm for cytokine signalling?* J Viral Hepat, 1994. **1**(2): p. 87-103.

和文要旨

ダニ媒介性脳炎ウイルス(TBEV)はフラビウイルス属に分類され、人に重篤な脳炎を起こす人獣共通感染症の原因ウイルスである。自然界において、*Ixodes* 属のマダニと、げっ歯類を中心とした哺乳類動物の間で本ウイルスの感染環が成立している。ヒトは主に感染マダニの吸血によってTBEVに感染する。ヨーロッパ諸国とロシアを中心に年間約 10,000 人の患者が報告されているが、TBEV の病原性メカニズムはほとんど明らかになっておらず、未だ生ワクチンやウイルス特異的な治療法はない。また本ウイルスにはヨーロッパ型、シベリア型、極東型の 3 つのサブタイプが存在し、それぞれ異なる病態を示す他、同一のサブタイプにおいても株間で異なる病原性を有する株が報告されている。しかしそれらの病原性の相違を決定するウイルス側因子は、現在特定されていない。

日本では北海道で患者が発生し、発生地域の犬の血液から Oshima 5-10 株が分離された。Oshima 株は、遺伝子解析により高病原性の極東型に分類されるが、ロシアの脳炎患者由来である標準株の Sofjin-HO 株よりもマウスに対する病原性は低い。その一方で両株の塩基配列の相同性は 95%以上と高いため、両株の病原性の相違に関わるウイルスの配列・領域を特定することで、極東型 TBEV の病態発現機序の解明に重要な知見が得られると考えられる。本研究では異なる病原性を持つ両株のキメラウイルスを作製し、病原性の相違に関わるウイルス側因子の特定とそれが関わる病態発現機構の解明を試みた。

本研究ではリバーシジェネティクス法を用いて、ウイルスゲノムの全領域を 11 の領域に分け、高病原性の Sofjin 株の各領域を低病原性の Oshima 株に導入後したキメラウイルスを作製した。これらのキメラウイルスをマウスに皮下接種し、その病原性を親株である Oshima 株と比較することで、病原性決定因子の特定を試みた。その結果、多くのキメラウイルスで病原性の上昇が見られ、一部の非構造蛋白、及び 5'-, 3'-非翻訳領域(UTR)などの、複数のウイルス側因子によって高い病原性が発現することが示唆された。その中でも、Sofjin 株の 3'-UTR variable region を導入

したキメラウイルスでは、最も著明な病原性の上昇が見られ、本領域が病原性に重要であることが明らかとなった。

3'-UTR variable region は株間での多様性が高く、ヒトの患者由来株や、実験室継代を繰り返した株においては、本領域における欠損や polyA の挿入が見られることが知られている。しかし病態発現機構における本領域の役割はまだ明らかにされていない。本研究における RNA 二次構造予測の結果、Oshima 株の 3'-UTR variable region 内には、6 つの stem loop (SL) 構造が存在することが分かった。このうち Sofjin 株では 2、3 及び 4 番目の SL 構造 (SL2、SL3 及び SL4) を欠損している。病態発現機構における本領域の役割を調べるため、Oshima 株から SL2 及び SL3 を含む領域、或いは SL4 を含む領域をそれぞれ欠損させた変異ウイルスを作製した。また他の株に見られる polyA 配列と病原性との関連性を明らかにするため、Sofjin 株の variable region の欠損箇所に、35 塩基の polyA 配列を挿入した Oshima 株由来の変異ウイルスを作製した。これらのウイルスと Sofjin 株及び Oshima 株をマウスに皮下接種し、病原性の比較解析を行なった。その結果、今回導入した variable region の変異により、いずれのウイルスの病原性も Sofjin 株と同程度に上昇する事が示された。その一方でマウス脳内での増殖性は、Sofjin 株と比較して、Oshima 株及び変異ウイルスで有意に低く、変異導入により脳内でのウイルス増殖性は上昇しなかった。本研究結果から、3'-UTR variable region への変異導入により、脳内でのウイルス増殖性の影響が認められないにも関わらず、マウスにおける病原性が上昇する事が明らかとなった。また本領域内の特定の RNA 配列や二次構造ではなく、本領域全体が構成する RNA の高次構造が病原性の相違に大きく関与していることが予想される。

3'-UTR が関連する病態発現機構として、3'-UTR から産生され、宿主のインターフェロン系に影響し、病原性に関与することが他のフラビウイルスで報告されている subgenomic flavivirus RNA (sfRNA) に着目した。TBEV の病態発現における sfRNA の病原性への関与及び variable region の部分的欠損による影響を調べるため、TBEV に感染させた培養細胞及び感染マウス脳より抽出した RNA を用い、本領域の部分的欠損或いは polyA の挿入の有無と sfRNA

の産生への影響を調べたところ、いずれのウイルスにおいても sfRNA の産生量や長さには相違はなく、病原性との関連性は見られなかった。これに加えて、Sofjin 株、Oshima 株及び変異ウイルス間で、感染時の培養細胞におけるインターフェロン (IFN)- β の誘導と IFN 応答遺伝子群の発現状況をレポーターアッセイにより比較したが、これらの発現と病原性との間にも、関連性は見られなかった。

以上の本研究結果から、極東型 TBEV の株間の病原性の相違は、複数のウイルス側因子によって決定され、その中でも特に 3'-UTR variable region が重要であることが明らかとなった。さらに variable region 全体が構成する高次構造が、未知の病態発現メカニズムを介して病原性に関与することが示唆された。これらの成績は、今後 TBEV をはじめとするフラビウイルスの病態発現機構の解明と、ワクチン及び予防・治療法の開発に大きく貢献するものと期待される。